Supporting Information for

Molecular Imaging of Peroxynitrite with HKGreen-4 in Live Cells and Tissues

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Contents

1. General Methods

All chemicals were purchased from Aldrich or Fluka, and used as received without further purification. All reactions were performed in oven-dried apparatus under an inert atmosphere (e.g, Ar or N_2) when necessary. All reagents and solvents for reactions were used as received from commercial sources unless otherwise stated. Dichloromethane, toluene, DMF, and pyridine were distilled from calcium hydride. THF and dioxane were distilled from sodium/benzophenone.

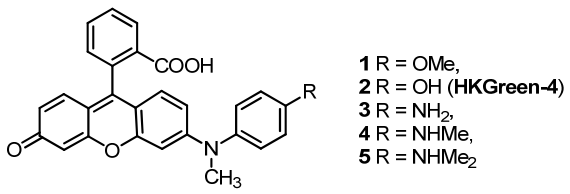
Air and moisture-sensitive compounds were introduced via syringes through rubber septa. Reactions were monitored by thin layer chromatography (TLC) using E. Merck silica gel 60 precoated glass plates with 0.25 mm thickness. Components were visualized by illumination with a short-wavelength ultra-violet light and/or staining in phosphomolybdic acid (PMA) or $KMnO_4$ solution followed by heating. Flash column chromatography was performed using the indicated solvents on E. Merck silica gel 60 (230−400 mesh ASTM).

Unless otherwise stated, NMR spectra were recorded in $CDCl_3$ or CD_3OD at ambient temperature on a Bruker Avance DPX 300 Fourier Transform Spectrometer operating at 300 MHz for ^1H and at 75.47 MHz for ^{13}C or Bruker Avance DPX 400 Fourier Transform Spectrometer operating at 400 MHz for ^1H and at 100.6 MHz for ^{13}C. ^1H NMR chemical shifts were reported using tetramethylsilane (TMS, δ 0.00 ppm) or CD_3OD (CD_3, δ 3.31 ppm) as internal standard. ^{13}C NMR chemical shifts were reported using the central line of $CDCl_3$ (δ 77.00 ppm) or CD_3OD (δ 49.00 ppm) as internal standard.

Mass spectra were recorded with a Finnigan MAT 95 mass spectrometer for both low resolution and high resolution analysis. HPLC analysis was performed with an Agilent 1100 HPLC system.

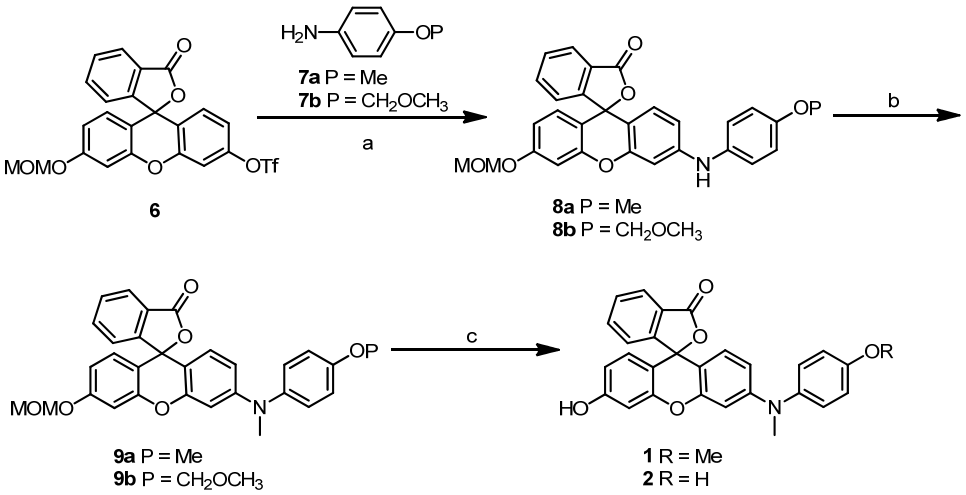
2. Syntheses of Probes

Scheme S1. Structures of the new series of rhodol-based fluorescent probes for detecting peroxynitrite



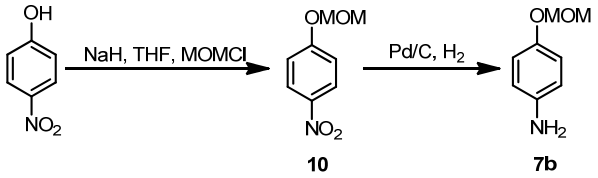
1 R = OMe,
2 R = OH (**HKGreen-4**)
3 R = NH_2,
4 R = NHMe,
5 R = $NHMe_2$

Scheme S2. Synthetic scheme for compounds **1** and **2**



Reagents and Conditions: (a) $Pd(OAc)_2$, BINAP, Cs_2CO_3, toluene, 100 °C, 20 h, 85% yield for **8a**, and 82% yield for **8b**; (b) NaH, THF, MeI, 12 h, 83% yield for **9a**, and 76% yield for **9b**; (c) TFA, CH_2Cl_2, 0 °C to rt, 2 h, 92% yield for **1**, and 90% yield for **2**.

*Synthesis of compound **7b***



To a solution of *p*-nitrophenol (1.39 g, 10 mmol) in THF (40 mL) was added NaH (0.6 g, 15 mmol, 60% in mineral oil) in small portions at 0 °C with vigorously stirring. After half an hour, MOMCl (1.2 mL, 15 mmol) was then introduced into the above suspension. The mixture was stirred at room temperature for 3 h and quenched with water. The resulting mixture was extracted with diethyl ether. The organic solution was then dried over anhydrous sodium sulfate and concentrated to provide the crude product **10** (1.80 g, 98% yield), which was directly used for the next step without further purification. Characterization data of **10**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.18 (d, J = 12.6 Hz, 2H), 7.11 (d, J = 12.6 Hz, 2H), 5.27 (s, 2H), 3.50 (s, 3H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 162.2, 125.7, 116.0, 103.3, 94.3, 56.4; LRMS (EI) m/z (%) 183 (M^+; 49), 137 (100); HRMS (EI) for $C_8H_9NO_4$ (M^+): calcd 183.0532, Found: 183.0529.

To a solution of **10** (1.80 g, 10 mmol) in MeOH was slowly added palladium (180 mg, 10% on activated carbon powder, 10% on the weight of **10**). The mixture was hydrogenated for 4 h at room temperature. The mixture was then filtered through a pad of Celite, and the filtrate was concentrated *in vacuo* to quantitatively give the product **7b** (1.50 g, 98% yield), which was directly used for the next step without further purification. Characterization data of **7b**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.87 (d, *J* = 8.8 Hz, 2H), 6.63 (d, *J* = 8.8 Hz, 2H), 5.07 (s, 2H), 3.47 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 150.2, 141.0, 117.8, 116.2, 95.5, 55.8.

*Synthesis of compound **8a***

Compound **8a** was synthesized from the coupling reaction between **6** and *p*-anisidine (**7a**, 1.2 equiv) in 85% yield according to a procedure reported previously.[1] Characterization data of **8a**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.03 (d, *J* = 7.4 Hz, 1H), 7.70 – 7.60 (m, 2H), 7.21 (d, *J* = 7.4 Hz, 1H), 7.14 (d, *J* = 8.9 Hz, 2H), 6.94 – 6.93 (m, 1H), 6.91 (d, *J* = 8.9 Hz, 2H), 6.73 – 6.71 (m, 3H), 6.60 (d, *J* = 8.6 Hz, 1H), 6.52 (dd, *J* = 8.6, 2.3 Hz, 1H), 5.79 (br, 1H), 5.20 (s, 2H), 3.84 (s, 3H), 3.49 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.4, 158.6, 156.1, 153.0, 152.5, 152.3, 147.8, 134.7 (CH), 133.7, 129.4 (CH), 128.9 (CH), 128.8 (CH), 126.8, 124.7 (CH), 123.9 (CH), 123.8 (CH), 116.0, 114.6 (CH), 112.5 (CH), 111.4 (CH), 108.7, 103.4 (CH), 100.4 (CH), 94.2 (CH_2), 83.8, 56.0 (CH_3), 55.4 (CH_3); LRMS (EI) *m/z* (%) 481 (M^+; 48), 437 (100); HRMS (EI) for $C_{29}H_{23}NO_6$ (M^+): calcd 481.1525, Found: 481.1520.

*Synthesis of compound **9a***

To a solution of **8a** (120 mg, 0.25 mmol) in THF (5 mL) at 0 ºC was added NaH (60% in mineral oil, 12 mg, 0.3 mmol). The suspension was stirred for half an hour and then MeI (30 μL, 0.50 mmol) was introduced. The mixture was stirred at room temperature overnight and then quenched with water. The mixture was diluted with ethyl acetate (20 mL), washed with 1N hydrochloric acid and brine. After dried over anhydrous sodium sulfate the organic solution was concentrated *in vacuo* and the residue was purified by silica gel column chromatography to give compound **9a** (103 mg, 83% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.99 (d, *J* = 7.4 Hz, 1H), 7.66 – 7.56 (m, 2H), 7.17 (d, *J* = 7.4 Hz, 1H), 7.12 (d, *J* = 8.9 Hz, 2H), 6.92 – 6.90 (m, 3H), 6.68 – 6.67 (m, 2H), 6.53 – 6.50 (m, 2H), 6.36 (dd, *J* = 8.6, 2.3 Hz, 1H), 5.18 (s, 2H), 3.82 (s, 3H), 3.46 (s, 3H), 3.27 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.4, 158.5, 157.3, 153.0, 152.5, 152.2, 151.4, 140.4, 134.6 (CH), 129.3 (CH), 128.9 (CH), 128.2 (CH), 127.8 (CH), 126.9, 124.7 (CH), 123.8 (CH), 116.3, 114.9 (CH), 114.6, 112.6, 112.4 (CH), 110.5 (CH), 107.1, 103.4 (CH), 100.0 (CH), 94.2 (CH_2), 83.7, 56.0 (CH_3), 55.3 (CH_3), 40.2 (CH_3); LRMS (EI) *m/z* (%) 495 (M^+; 35), 452 (100); HRMS (EI) for $C_{30}H_{25}NO_6$ (M^+): calcd 495.1682, Found: 495.1685.

*Synthesis of compound **1***

To a solution of **9a** (103 mg, 0.21 mmol) in dry CH_2Cl_2 (3 mL) was added trifluoroacetic acid (3 mL) dropwise at 0 ºC. The resulting solution was stirred at room temperature until TLC indicated all starting materials were consumed. The mixture was then concentrated *in vacuo* and azeotroped with toluene three times. The residue was dissolved in ethyl acetate and washed with saturated $NaHCO_3$, followed by water and brine. The organic solution was concentrated and the resulting residue was purified by silica gel column chromatography to give compound **1** (87 mg, 92% yield). ^{1}H NMR (400 MHz, CD_3OD) δ 8.33 (d, *J* = 7.5 Hz, 1H), 7.86 – 7.80 (m, 2H), 7.41 (d, *J* = 7.5 Hz, 1H), 7.28 (d, *J* = 8.9 Hz, 2H), 7.23 – 7.14 (m, 3H), 7.09 (d, *J* = 8.9 Hz, 2H), 7.00 – 6.93 (m, 3H), 3.83 (s, 3H), 3.61 (s, 3H); ^{13}C NMR (100 MHz, CD_3OD) δ 167.4, 166.6, 159.5, 158.6, 158.3, 156.9, 148.2, 136.9, 134.9, 132.6, 131.0, 130.9, 130.5, 130.2, 129.2, 127.2, 123.3, 116.9, 116.7, 115.2, 115.0, 114.8, 101.7, 97.2, 54.5, 40.8; LRMS (EI) *m*/*z* (%) 451 (M^+; 23), 406 (100); HRMS (EI) for $C_{28}H_{21}NO_5$ (M^+): calcd 451.1420, Found: 451.1425.

*Synthesis of compound **8b***

An oven-dried Schlenk tube was charged with $Pd(OAc)_2$ (4 mg, 0.02 mmol), BINAP (18 mg, 0.03 mmol) and Cs_2CO_3 (91 mg, 0.28 mmol), and flushed with Ar gas for 5 min. A solution of **6** (102 mg, 0.2 mmol) and 4-(methoxymethoxy)aniline **7b** (37 mg, 0.24 momol) in toluene (2 mL) was added, and the resulting mixture was first stirred under Ar at room temperature for 30 min and then at 100 ºC for 20 h. The reaction mixture was allowed to cool to room temperature, diluted with CH_2Cl_2 and filtered through a pad of Celite. The filter cake was washed with CH_2Cl_2 (3 × 10 mL). The filtrate was then concentrated and the residue was purified by silica gel column chromatography to give compound **8b** (84 mg, 82% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.99 (d, *J* = 7.4 Hz, 1H), 7.66 – 7.58 (m, 2H), 7.16 (d, *J* = 7.4 Hz, 1H), 7.08 (d, *J* = 8.9 Hz, 2H), 6.99 (d, *J* = 8.9 Hz, 2H), 6.91 (s, 1H), 6.73 (s, 1H), 6.67 (s, 2H), 6.57 – 6.48 (m, 2H), 5.94 (s, br, 1H), 5.16 (s, 2H), 5.14 (s, 2H), 3.48 (s, 3H), 3.45 (s, 3H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 169.6, 158.8, 153.5, 153.1, 152.6, 152.5, 147.6, 135.3, 134.9, 129.6, 129.1, 129.0, 127.0, 124.9, 124.0, 123.2, 117.4, 112.7, 112.6, 111.9, 109.0, 103.6, 100.8, 94.9, 94.3, 83.8, 56.1, 56.0; LRMS (EI) *m*/*z* (%) 511 (M^+; 47), 467 (100); HRMS (EI) for $C_{30}H_{25}NO_7$ (M^+): calcd 511.1631, Found: 511.1636.

*Synthesis of compound **9b***

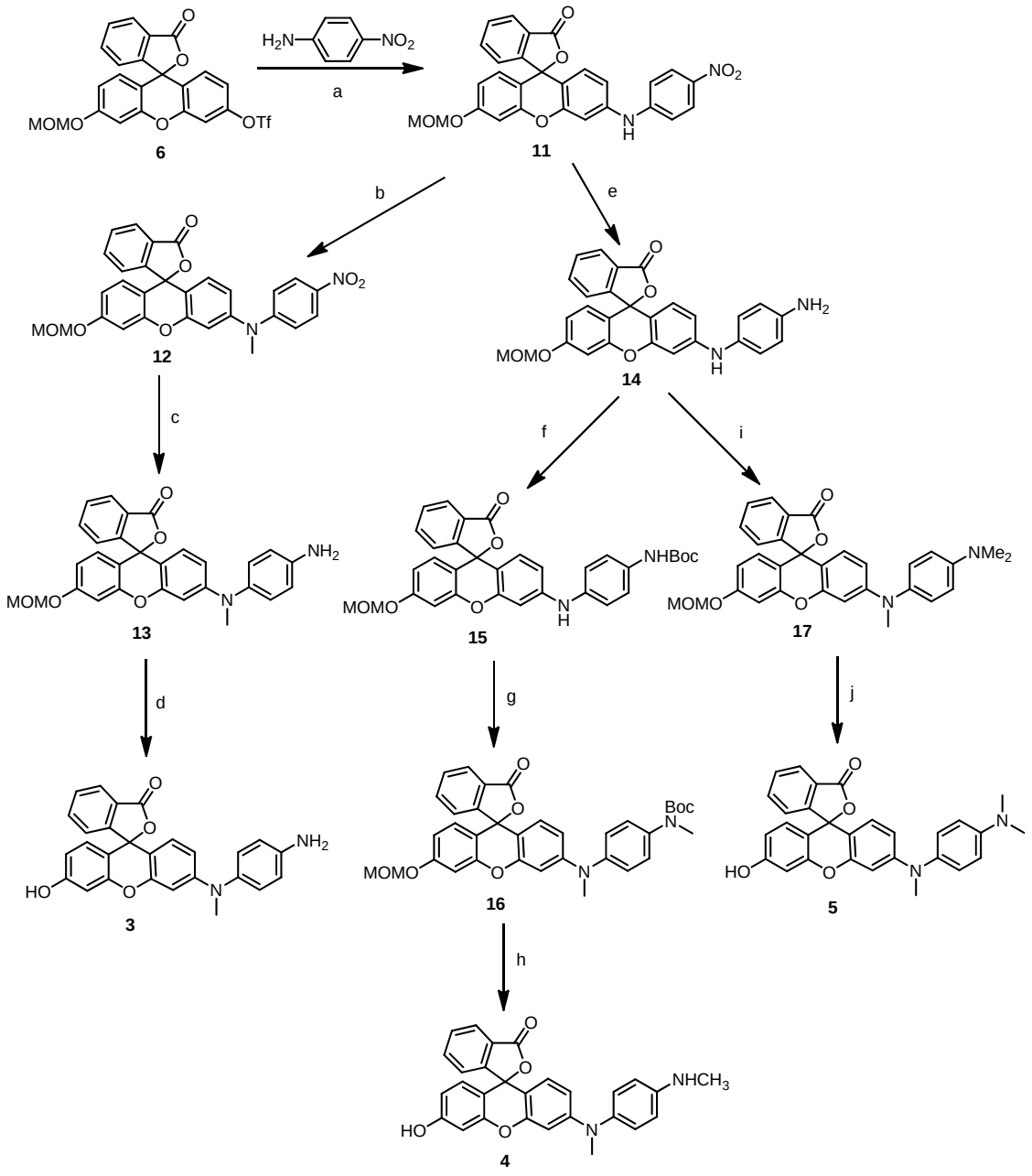
To a solution of **8b** (84 mg, 0.16 mmol) in THF (4 mL) at 0 ºC was added NaH (10 mg, 0.24 mmol, 60% in mineral oil). The suspension was stirred for half an hour and then MeI (20 μL, 0.32

mmol) was introduced. The mixture was stirred at room temperature overnight and then quenched with water. The mixture was diluted with ethyl acetate, washed with 1N hydrochloric acid and brine. After dried over anhydrous sodium sulfate the organic solution was concentrated *in vacuo* and the residue was purified by silica gel column chromatography to give compound **9b** (64 mg, 76% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.98 (d, *J* = 7.2 Hz, 1H), 7.66 – 7.58 (m, 2H), 7.15 (d, *J* = 7.2 Hz, 1H), 7.14 – 7.08 (m, 2H), 7.04 (d, *J* = 9.0 Hz, 2H), 6.92 (s, 1H), 6.67 (s, 1H), 6.52 (d, *J* = 9.0 Hz, 2H), 6.36 – 6.34 (m, 2H), 5.16 (s, 4H), 3.48 (s, 3H), 3.45 (s, 3H), 3.25 (s, 3H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 169.5, 158.7, 155.0, 153.1, 152.6, 152.4, 151.5, 141.7, 134.8, 129.5, 129.1, 128.4, 127.7, 127.1, 124.8, 124.0, 117.5, 112.8, 112.6, 110.9, 107.5, 103.6, 100.5, 94.6, 94.3, 83.8, 56.1, 56.0, 40.4; LRMS (EI) *m/z* (%) 525 (M^+; 52), 481 (78), 436 (100); HRMS (EI) for $C_{31}H_{27}NO_7$ (M^+): calcd 525.1788, Found: 525.1795.

*Synthesis of compound **2***

To a solution of **9b** (64 mg, 0.12 mmol) in dry CH_2Cl_2 (2 mL) was added trifluoroacetic acid (2 mL) dropwise at 0 ºC. The resulting solution was stirred at room temperature until TLC indicated all starting materials were consumed. The mixture was then concentrated *in vacuo* and azeotroped with toluene three times. The residue was dissolved in ethyl acetate and washed with saturated $NaHCO_3$, followed by water and brine. The organic solution was concentrated *in vacuo* and then the residue was purified by silica gel column chromatography to give compound **2** (47 mg, 90% yield). ^{1}H NMR (500 MHz, CD_3OD) δ 8.26 (d, *J* = 7.7 Hz, 1H), 7.83 – 7.75 (m, 2H), 7.35 (d, *J* = 7.7 Hz, 1H), 7.13 (d, *J* = 8.7 Hz, 2H), 7.09 (d, *J* = 9.0 Hz, 1H), 7.04 – 7.02 (m, 2H), 6.92 – 6.88 (m, 4H), 6.80 (d, *J* = 9.4 Hz, 1H), 3.52 (s, 3H); ^{13}C NMR (125.8 MHz, CD_3OD) δ 169.1, 168.2, 159.3, 159.1, 158.7, 158.0, 139.2, 138.1, 134.6, 132.3, 132.1, 131.7, 131.6, 131.3, 130.1, 130.0, 128.9, 118.1, 118.0, 117.4, 115.9, 115.4, 103.6, 99.1, 42.3; LRMS (EI) *m/z* (%) 437 (M^+; 16), 392 (100); HRMS (EI) for $C_{27}H_{19}NO_5$ (M^+): calcd 437.1263, Found: 437.1266.

Scheme S3. Synthetic scheme for compounds **3–5**



Reagents and Conditions: (a) $Pd(OAc)_2$, BINAP, Cs_2CO_3, toluene, 100 °C, 20 h, 80% yield; (b) NaH, THF, MeI, 12 h, 83% yield; (c) Pd/C, H_2, EtOH, 2 h, 77%; (d) TFA, CH_2Cl_2, 0 °C to rt, 2 h, 91% yield; (e) Pd/C, H_2, EtOH, 2 h, 74%; (f) Boc_2O, Et_3N, DMAP, CH_2Cl_2, overnight, 96%; (g) NaH, THF, MeI, 12 h, 67% yield; (h) TFA, CH_2Cl_2, 0 °C to rt, 2 h, 88% yield; (i) NaH, THF, MeI, 12 h, 56% yield; (j) TFA, CH_2Cl_2, 0 °C to rt, 2 h, 93% yield.

*Synthesis of compound **11***

Compound **11** was synthesized from the coupling reaction between **6** and *p*-nitroaniline (1.2 equiv) in 80% yield according to a procedure reported previously.[1] Characterization data of compound **11**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.06 – 8.01 (m, 3H), 7.72 – 7.63 (m, 2H), 7.19 (d, J = 7.2 Hz, 1H), 7.06 – 7.03 (m, 3H), 6.93 (s, 1H), 6.80 – 6.64 (m, 4H), 5.18 (s, 2H), 3.46 (s, 3H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 169.8, 159.0, 152.8, 152.2, 148.8, 142.6, 140.3, 135.4, 130.0, 129.1, 129.0, 126.5, 126.0, 125.1, 124.0, 115.9, 115.1, 113.2, 112.0, 106.9, 103.7, 94.3, 83.4, 56.2; LRMS (EI) m/z (%) 452 ($M^+ - CO_2$; 10), 136 (100); LRMS (FAB) m/z (%) 498 (M^+; 25), 154 (100); HRMS (EI) for $C_{27}H_{20}N_2O_5$ ($M^+ - CO_2$): calcd 452.1372, Found: 452.1366.

*Synthesis of compound **12***

Compound **12** was synthesized from *N*-methylation of **11** in a similar procedure as the syntheses of **9a** and **9b**. The yield for this transformation is 83%. Characterization data of compound **12**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.10 – 8.04 (m, 3H), 7.80 – 7.63 (m, 2H), 7.24 (d, J = 7.4 Hz, 1H), 7.14 (d, J = 2.0 Hz, 1H), 6.98 (d, J = 2.0 Hz, 1H), 6.90 – 6.82 (m, 4H), 6.76 – 6.73 (m, 2H), 5.20 (s, 2H), 3.48 (s, 3H), 3.44 (s, 3H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 159.0, 153.0, 152.7, 152.4, 152.1, 148.5, 139.4, 135.2, 130.0, 129.6, 129.1, 126.6, 125.7, 125.2, 124.0, 120.8, 116.5, 114.5, 113.3 (2C), 112.1, 103.7, 94.4, 85.3, 56.2, 40.4; LRMS (EI) m/z (%) 466 ($M^+ - CO_2$; 11), 341 (96), 267 (100); LRMS (FAB) m/z (%) 511 (M^+; 38), 342 (60), 109 (100); HRMS (EI) for $C_{28}H_{22}N_2O_5$ ($M^+ - CO_2$): calcd 466.1522, Found: 466.1529.

*Synthesis of compound **13***

To a solution of **12** (67 mg, 0.13 mmol) in EtOH (10 mL) was slowly added palladium (10% on activated carbon powder, 7 mg). The mixture was hydrogenated for 2 hours at room temperature. The mixture was then filtered through a pad of Celite, and the filtrate was concentrated *in vacuo*. The residue was purified by silica gel column chromatography to give compound **13** (48 mg, 77% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.99 (d, J = 7.5 Hz, 1H), 7.64 – 7.57 (m, 2H), 7.17 (d, J = 7.5 Hz, 1H), 6.98 (d, J = 8.6 Hz, 2H), 6.91 (s, 1H), 6.710 – 6.67 (m, 4H), 6.51 – 6.48 (m, 2H), 6.35 – 6.32 (m, 1H), 5.18 (s, 2H), 3.68 (br, 2H), 3.47 (s, 3H), 3.24 (s, 3H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 169.6, 158.7, 153.2, 152.7, 152.4, 151.8, 144.5, 138.7, 134.7, 129.4, 129.1, 128.3, 128.0, 127.2, 124.8, 124.0, 116.2, 112.8, 112.5, 110.5, 106.8, 103.6, 99.8, 94.4, 84.0, 56.1, 40.4; LRMS (EI) m/z (%) 480 (M^+; 68), 422 (100); HRMS (EI) for $C_{29}H_{24}N_2O_5$ (M^+): calcd 480.1685, Found: 480.1684.

*Synthesis of compound **3***

The compound **3** was synthesized by treatment of **13** with TFA as shown above. The compound **3** was obtained in 91% yield. ^{1}H NMR (400 MHz, CD_3OD) δ 8.02 (d, *J* = 7.2 Hz, 1H), 7.70 – 7.64 (m, 2H), 7.16 (d, *J* = 7.2 Hz, 1H), 6.92 (d, *J* = 8.6 Hz, 2H), 6.76 (d, *J* = 8.6 Hz, 2H), 6.69 (d, *J* = 8.8 Hz, 1H), 6.64 (d, *J* = 2.2 Hz, 1H), 6.59 – 6.52 (m, 3H), 6.42 (dd, *J* = 8.8, 2.2 Hz, 1H), 3.26 (s, 3H); ^{13}C NMR (100 MHz, CD_3OD) δ 164.6, 154.5, 153.7, 153.6, 153.5, 147.5, 146.2, 137.3, 133.7, 129.5, 129.4, 128.5, 127.6, 127.3, 125.9, 125.4, 116.1, 114.6, 114.1, 111.4, 108.5, 102.3, 98.6, 76.0, 39.7; LRMS (EI) *m/z* (%) 436 (M^+; 36), 390 (100); HRMS (EI) for $C_{27}H_{20}N_2O_4$ (M^+): calcd 436.1423, Found: 436.1423.

*Synthesis of compound **14***

To a solution of **11** (200 mg, 0.40 mmol) in EtOH (20 mL) was slowly added palladium (10% on activated carbon powder, 20 mg). The mixture was hydrogenated for 2 hours at room temperature. The mixture was then filtered through a pad of Celite, and the filtrate was concentrated *in vacuo*. The residue was purified by silica gel column chromatography to give compound **14** (138 mg, 74% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.99 (d, *J* = 7.3 Hz, 1H), 7.64 – 7.57 (m, 2H), 7.16 (d, *J* = 7.3 Hz, 1H), 6.96 (d, *J* = 8.6 Hz, 2H), 6.89 (d, *J* = 1.3 Hz, 1H), 6.710 – 6.67 (m, 4H), 6.51 – 6.48 (m, 2H), 6.35 – 6.32 (m, 1H), 5.18 (s, 2H), 3.68 (br, 2H), 3.47 (s, 3H), 3.24 (s, 3H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 169.6, 158.7, 153.2, 152.7, 152.4, 151.8, 144.5, 138.7, 134.7, 129.4, 129.1, 128.3, 128.0, 127.2, 124.8, 124.0, 116.2, 112.8, 112.5, 110.5, 106.8, 103.6, 99.8, 94.4, 84.0, 56.1, 40.4; LRMS (EI) *m/z* (%) 466 (M^+; 70), 422 (100); HRMS (EI) for $C_{28}H_{22}N_2O_5$ (M^+): calcd 466.1529, Found: 466.1521.

*Synthesis of compound **15***

To a solution of **14** (90 mg, 0.19 mmol) in dry CH_2Cl_2 (5 mL) were added Et_3N (30 μL, 0.21 mmol), DMAP (5 mg, 0.04 mmol), and Boc_2O (50 μL, 0.21 mmol) successively at room temperature. The reaction mixture was stirred overnight and then diluted with CH_2Cl_2 (30 mL). The resulting solution was washed with saturated $NaHCO_3$ solution followed by 0.1 N HCl and brine. The organic layer was dried over anhydrous sodium sulfate, concentrated and purified by silica gel column chromatography to give compound **15** (103 mg, 96% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.99 (d, *J* = 7.4 Hz, 1H), 7.66 – 7.57 (m, 2H), 7.30 (d, *J* = 8.8 Hz, 2H), 7.16 (d, *J* = 7.4 Hz, 1H), 7.05 (d, *J* = 8.8 Hz, 2H), 6.91 (d, *J* = 2.0 Hz, 1H), 6.76 (d, *J* = 2.0 Hz, 1H), 6.70 – 6.65 (m, 2H), 6.61 (br, 1H), 6.57 – 6.51 (m, 2H), 5.98 (br, 1H), 5.17 (s, 2H), 3.46 (s, 3H), 1.51 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.6, 158.7, 153.1, 153.0, 152.5, 147.0, 136.4, 134.9, 133.8, 129.6, 129.0, 128.9, 126.9, 124.9, 124.0,

121.8, 120.1, 112.7, 112.6, 112.2, 109.3, 106.8, 103.6, 101.3, 94.3, 83.7, 80.4, 56.1, 28.4; LRMS (EI) *m/z* (%) 466 ([M+H]$^+$ – Boc, 8), 153 (60); HRMS (EI) for $C_{28}H_{22}N_2O_5$ ([M+H]$^+$ – Boc): calcd 466.1529, Found: 466.1530.

*Synthesis of compound **16***

To a solution of **15** (103 mg, 0.18 mmol) in THF (4 mL) at 0 ºC was added NaH (16 mg, 0.40 mmol, 60% in mineral oil). The suspension was stirred for half an hour and then MeI (34 μL, 0.54 mmol) was introduced. The mixture was stirred at room temperature overnight and then quenched with water. The mixture was diluted with ethyl acetate (30 mL), washed with 1N hydrochloric acid and brine. After dried over anhydrous sodium sulfate the organic solution was concentrated *in vacuo* and the residue was purified by silica gel column chromatography to give compound **16** (72 mg, 67% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.00 (d, *J* = 7.4 Hz, 1H), 7.67 – 7.57 (m, 2H), 7.23 (d, *J* = 8.4 Hz, 2H), 7.18 (d, *J* = 7.4 Hz, 1H), 7.13 (d, *J* = 8.4 Hz, 2H), 6.93 (s, 1H), 6.71 – 6.64 (m, 3H), 6.55 (d, *J* = 8.8 Hz, 1H), 6.49 (d, *J* = 8.8 Hz, 1H), 5.18 (s, 2H), 3.46 (s, 3H), 3.31 (s, 3H), 3.26 (s, 3H), 1.47 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.6, 158.7, 154.8, 153.1, 152.6, 152.4, 150.9, 144.8, 140.4, 134.8, 129.5, 129.1, 128.4, 127.0, 126.6, 125.3, 124.9, 124.0, 112.7, 112.6, 112.2, 108.6, 103.6, 102.1, 94.3, 83.6, 80.4, 56.1, 40.2, 37.3, 28.3; LRMS (EI) *m/z* (%) 595 ([M+H]$^+$; 20), 539 (14), 495 (100); HRMS (EI) for $C_{35}H_{34}N_2O_7$ (M$^+$): calcd 594.2366, Found: 594.2361.

*Synthesis of compound **4***

The compound **4** was synthesized by treatment of **16** with TFA as shown above. The compound **4** was obtained in 88% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.05 (s, 1H), 7.56 (s, 2H), 7.11 (s, 1H), 6.92 (s, 2H), 6.73 – 6.68 (m, 3H), 6.59 – 6.41 (m, 5H), 3.24 (s, 3H), 2.82 (s, 3H); ^{13}C NMR (125.8 MHz, $CDCl_3$) δ 170.0, 163.2, 154.4, 153.9, 147.8, 146.3, 136.4, 133.1, 130.7, 129.8, 129.5, 129.3, 127.7, 127.4, 126.8, 125.8, 114.8, 113.5, 112.2, 109.7, 102.9, 98.9, 40.8, 30.9; LRMS (EI) *m/z* (%) 450 (M$^+$; 30), 405 (100); HRMS (EI) for $C_{28}H_{22}N_2O_4$ (M$^+$): calcd 450.1580, Found: 450.1584.

*Synthesis of compound **17***

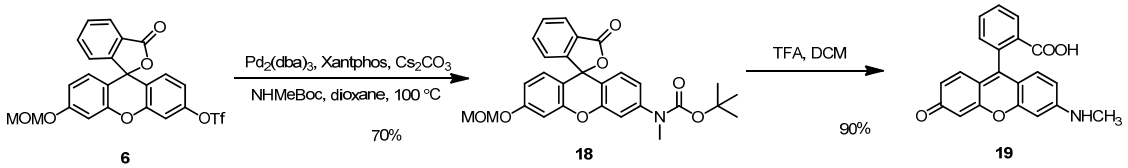
To a solution of **14** (52 mg, 0.11 mmol) in THF (3 mL) at 0 ºC was added NaH (18 mg, 0.44 mmol, 60% in mineral oil). The suspension was stirred for half an hour and then MeI (28 μL, 0.44 mmol) was introduced. The mixture was stirred at room temperature overnight and then quenched with water. The mixture was diluted with ethyl acetate (20 mL), washed with 1N hydrochloric acid and brine. After dried over anhydrous sodium sulfate the organic solution was concentrated *in vacuo* and

the residue was purified by silica gel column chromatography to give compound **17** (31 mg, 56% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.99 (d, *J* = 7.4 Hz, 1H), 7.65 – 7.56 (m, 2H), 7.17 (d, *J* = 7.4 Hz, 1H), 7.06 (d, *J* = 8.9 Hz, 2H), 6.91 (s, 1H), 6.74 (d, *J* = 8.9 Hz, 2H), 6.67 (s, 2H), 6.51 – 6.48 (m, 2H), 6.35 – 6.33 (m, 1H), 5.18 (s, 2H), 3.47 (s, 3H), 3.26 (s, 3H), 2.96 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.6, 158.6, 153.2, 152.7, 152.4, 152.0, 148.8, 136.9, 134.7, 129.4, 129.1, 128.3, 127.7, 127.1, 124.8, 124.0, 113.5, 112.8, 112.4, 110.4, 106.6, 103.6, 99.6, 94.3, 84.1, 56.1, 40.7, 40.4; LRMS (EI) *m*/*z* (%) 509 ([M+H]$^+$; 100); HRMS (EI) for $C_{31}H_{28}N_2O_5$: calcd 508.1998, Found: 508.2000.

*Synthesis of compound **5***

The compound **5** was synthesized by treatment of **17** with TFA as shown above. The compound **5** was obtained in 93% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.04 (d, *J* = 7.1 Hz, 1H), 7.60 – 7.54 (m, 2H), 7.13 (d, *J* = 7.1 Hz, 1H), 7.03 (d, *J* = 8.6 Hz, 2H), 6.72 (d, *J* = 8.6 Hz, 2H), 6.69 – 6.63 (m, 3H), 6.40 (s, 2H), 6.39 (d, *J* = 9.3 Hz, 1H), 3.60 (br, 3H), 3.26 (s, 3H), 2.96 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.1, 162.5, 154.1, 153.8, 153.2, 149.0, 147.8, 136.1, 133.4, 130.0, 129.7, 129.4, 129.0, 127.6, 126.2, 125.3, 114.2, 113.5, 112.1, 111.6, 108.7, 103.0, 99.1, 40.7, 40.6; LRMS (EI) *m*/*z* (%) 464 (M^+; 35), 419 (100); HRMS (EI) for $C_{29}H_{24}N_2O_4$ (M^+): calcd 464.1736, Found: 464.1738.

Scheme S4. Synthesis of *N*-methylrhodol



*Synthesis of compound **18***

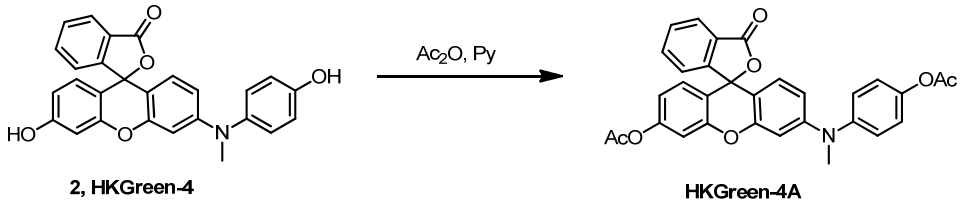
An oven-dried Schlenk tube was charged with $Pd_2(dba)_3$ (26 mg, 0.029 mmol), Xantphos (33 mg, 0.06 mmol) and Cs_2CO_3 (113 mg, 0.35 mmol), and flushed with Ar gas for 5 min. A solution of **6** (145 mg, 0.29 mmol) and *t*-butyl *N*-methylcarbamate (75 mg, 0.57 momol) in dioxane (3 mL) was added, and the resulting mixture was first stirred under Ar at room temperature for 30 min and then at 100 °C for 20 h. The reaction mixture was allowed to cool to room temperature, diluted with CH_2Cl_2 and filtered through a pad of Celite. The filter cake was washed with CH_2Cl_2 (3 × 10 mL). The filtrate was then concentrated and the residue was purified by silica gel column chromatography to give compound **18** (98 mg, 0.21 mmol, 70% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.03 (d, *J* = 7.2 Hz, 1H), 7.70 – 7.59 (m, 2H), 7.21 (d, *J* = 2.1 Hz, 1H), 7.17 (d, *J* = 7.2 Hz, 1H), 7.00 – 6.93 (m, 2H), 6.76 – 6.69

(m, 3H), 5.20 (s, 2H), 3.48 (s, 3H), 3.28 (s, 3H), 1.48 (s, 9H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 169.46, 159.02, 154.35, 153.16, 152.37, 151.39, 145.83, 135.14, 129.89, 129.16, 128.49, 128.04, 126.65, 125.16, 124.09, 120.69, 115.55, 113.11, 112.32, 103.75, 94.44, 82.68, 81.16, 56.29, 37.08, 28.40; LRMS (EI) *m/z* (%): 489 (M^+, 2), 445 (13), 389 (61); HRMS (EI): calcd for $C_{28}H_{27}NO_7$ (M^+), 489.1782; found, 489.1779.

*Synthesis of compound **19***

To a solution of **18** (75 mg, 0.15 mmol) in dry CH_2Cl_2 (2 mL) was added TFA (2 mL) dropwise at 0 °C. The resulting solution was stirred at room temperature for 2 hr. The mixture was concentrated *in vacuo* and then diluted with saturated $NaHCO_3$ solution. The mixture was extracted with chloroform containing 10% isopropanol three times. The organic layers were combined and dried over anhydrous sodium sulfate. The organic solution was concentrated and purified by silica gel column chromatography to give the product *N*-methylrhodol **19** (59 mg, 90% yield). ^{1}H NMR (400 MHz, CD_3OD) δ 8.22 (d, *J* = 7.4 Hz, 1H), 7.82 – 7.73 (m, 2H), 7.33 (d, *J* = 7.4 Hz, 1H), 7.04 (d, *J* = 9.0 Hz, 1H), 6.99 – 6.94 (m, 2H), 6.82 (dd, *J* = 9.0, 2.3 Hz, 1H), 6.77 – 6.74 (m, 2H), 3.01 (s, 3H); ^{13}C NMR (101 MHz, CD_3OD) δ 170.20, 168.12, 168.10, 159.65, 159.41, 157.53, 134.26, 132.84, 132.08, 131.96, 131.47, 131.39, 130.62, 129.69, 117.75, 117.23, 115.27, 114.38, 103.61, 96.42, 30.50; LRMS (EI) *m/z* (%): 301 (M^+ - CO_2, 23), 272 (11); HRMS (EI): calcd for $C_{20}H_{15}NO_2$ (M^+ - CO_2), 301.1097; found, 301.1082.

Scheme S5. Synthesis of probe **HKGreen-4A**



2, HKGreen-4 **HKGreen-4A**

To a solution of **2** (53 mg, 0.39 mmol) in pyridine (2 mL) was added acetic anhydride (1 mL). The resulting mixture was heated to reflux for 2 hours. Then the reaction mixture was quenched with water and diluted with ethyl acetate. The organic solution was washed with saturated $NaHCO_3$ and brine, dried over anhydrous sodium sulfate and concentrated. The residue was purified by silica gel column chromatography to give **HKGreen-4A** (46 mg, 73% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.99 (d, *J* = 7.3 Hz, 1H), 7.69 – 7.55 (m, 2H), 7.20 – 7.16 (m, 3H), 7.14 – 7.04 (m, 3H), 6.82 – 6.72 (m, 2H), 6.66 (d, *J* = 2.2 Hz, 1H), 6.56 (d, *J* = 8.8 Hz, 1H), 6.48 (dd, *J* = 8.8, 2.2 Hz, 1H), 3.29 (s, 3H), 2.28 (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 169.37, 169.21, 168.76, 152.73, 152.03, 151.96, 151.71, 150.84,

147.34, 145.03, 134.91, 129.66, 128.89, 128.35, 126.61, 126.13, 124.84, 123.92, 122.65, 117.07, 116.78, 112.24, 110.10, 108.20, 101.87, 82.82, 40.21, 20.97; LRMS (EI) *m/z* (%): 522 (M^+, 25), 478 (100); HRMS (EI): calcd for $C_{31}H_{23}NO_7$ (M^+), 521.1475; found, 521.1480.

3. General Protocols for Photophysical Characterization of Probes

The compounds **1–5** were dissolved in DMF to make 10 mM or 1 mM stock solutions, which were diluted 1000 times to 10 μM or 1 μM as testing solutions with 0.1 mM phosphate buffer (0.1 M, pH 7.4). The absorbance and fluorescence spectra of probe testing solutions were recorded under a CARY 50 Bio UV-Visible spectrophotometer and a Hitachi F-2500 fluorescence spectrophotometer, respectively. For the fluorescence spectra, slit widths were set at 2.5 nm for both excitation and emission spectra, and the photomultiplier voltage was 700 V.

To test the fluorescence responses of probes toward various ROS and RNS, aliquots of ROS or RNS solutions were slowly added to the testing solutions of probes (each 5 mL) with vigorously stirring at room temperature in the dark. The volume changes after addition of ROS or RNS solutions were less than 1%. The fluorescence intensities of the testing solutions were measured after 30 min or 1 h.

To determine the quantum yields, probe or fluorophore stock solutions (**2** and **19**) were prepared by accurately weighing and dissolving the samples in CH_3CN (HPLC grade). Testing solutions for measuring UV absorbance and fluorescence were prepared by further dilution of the stock solutions with 50 mM potassium phosphate buffer at pH 8.0. The quantum yields of the fluorophores were estimated by comparison of the integrated area of the corrected emission spectrum of the sample with that of a reference solution, i.e., a solution of fluorescein in 0.1 M NaOH solution (Φ = 0.95). The quantum yield of a sample was related to that of the reference, and determined by the equation

$$\Phi_{\text{sample}} = \left(\frac{A_{\text{reference}}}{A_{\text{sample}}}\right)\left(\frac{F_{\text{sample}}}{F_{\text{reference}}}\right)\Phi_{\text{reference}} \quad (1)$$

wherein Φ is the fluorescence quantum yield, A is the absorbance at the excitation wavelength, F is the area under the emission curve. The concentration of the reference was adjusted to match the absorbance of the test sample at the wavelength of excitation so that the absorbance ratio is equal to 1.

Sources for different ROS/RNS are described as follows. ROO$^{\bullet}$ was generated from 2,2'-Azobis(2-amidinopropane)dihydrochloride, which was firstly dissolved in deionizer water and then added into the probe testing solutions at 37 °C for 1 h. $^1O_2$ (singlet oxygen) was generated from 3,3'-(naphthalene-1,4-diyl)dipropionic acid. H_2O_2 solution was added directly. The stock H_2O_2 solution was purchased from Sigma-Aldrich. The concentration of H_2O_2 was determined by iodometric titration prior to use. $^{\bullet}$NO was generated from SNP (Sodium Nitroferricyanide (III) Dihydrate). The experiments were performed under anaerobic conditions. Deionizer water was degassed with Ar for 20 min. SNP was added into degassed deionizer water under Ar atmosphere then stirred for 30 min at 25 °C. The probe solution was also degassed before the reaction with SNP. Superoxide ($O_2^{\bullet-}$) was generated from xanthine/xanthine oxidase system. Xanthine oxidase was added first. After xanthine oxidase was dissolved, xanthine in 1.6 M NaOH was then added. The mixtures were stirred at 25 °C for 1 h. The source of NaOCl was commercial bleach. The concentration of OCl^- was determined by titration with $S_2O_3^{2-}$. Hydroxyl radical ($^{\bullet}$OH) was generated by Fenton reaction. To generate $^{\bullet}$OH, ferrous chloride was added in the presence of 10 equiv of H_2O_2. The concentration of $^{\bullet}$OH was equal to the Fe(II) concentration. Nitrogen dioxide (NO_2) was purchased from Aldrich and introduced into the probe solution via a gas tight syringe.[2] Carbonate radical ($CO_3^{\bullet-}$) was generated by the SOD1/H_2O_2/bicarbonate system.[3] Peroxynitrite ($ONOO^-$) solution was synthesized according to literature report. Briefly, a mixture of sodium nitrite (0.6 M) and hydrogen peroxide (0.7 M) was acidified with hydrochloric acid (0.6 M), and sodium hydroxide (1.5 M) was added within 1−2 s to make the solution alkaline. The excess hydrogen peroxide was removed by passing the solution through a short column of manganese dioxide. The resulting solution was split into small aliquots and stored at lower than −18 °C. The aliquots were thawed immediately before use, and the concentration of peroxynitrite was determined by measuring the absorption of the solution at 302 nm. The extinction coefficient of peroxynitrite solution in 0.1 M NaOH is 1670 M^{-1} cm^{-1} at 302 nm. $C_{ONOO-} = Abs_{302nm} / 1.67$ (mM).

4. Probe Screening

The responses of compound **1** and compound **2** (**HKGreen-4**) toward peroxynitrite are compared in Figure S1. Compound **1** shows a much weaker fluorescence increase toward peroxynitrite than compound **2**. Different reactivity of compounds **2**–**5** toward peroxynitrite and hypochlorous acid is summarized in Figure S2. Among these compounds, compound **2** shows good selectivity toward peroxynitrite over hypochlorous acid, while compounds **3**–**5** exhibit strong fluorescence responses toward both hypochlorous acid and peroxynitrite.

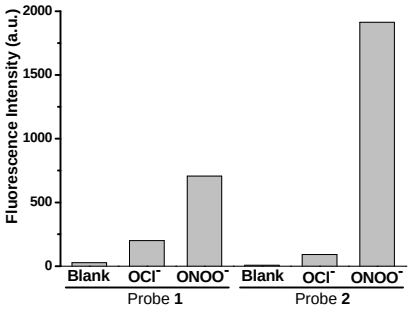


Figure S1. Fluorescence increases of compound **1** (10 μM in 0.1 M phosphate buffer at pH 7.4) and compound **2** (1 μM in 0.1 M phosphate buffer at pH 7.4) toward hypochlorous acid and peroxynitrite. Hypochlorous acid and peroxynitrite were both added in 1 equiv relative to compounds **1** and **2**. For both compounds the fluorescence intensities were recorded at 535 nm with excitation at 517 nm.

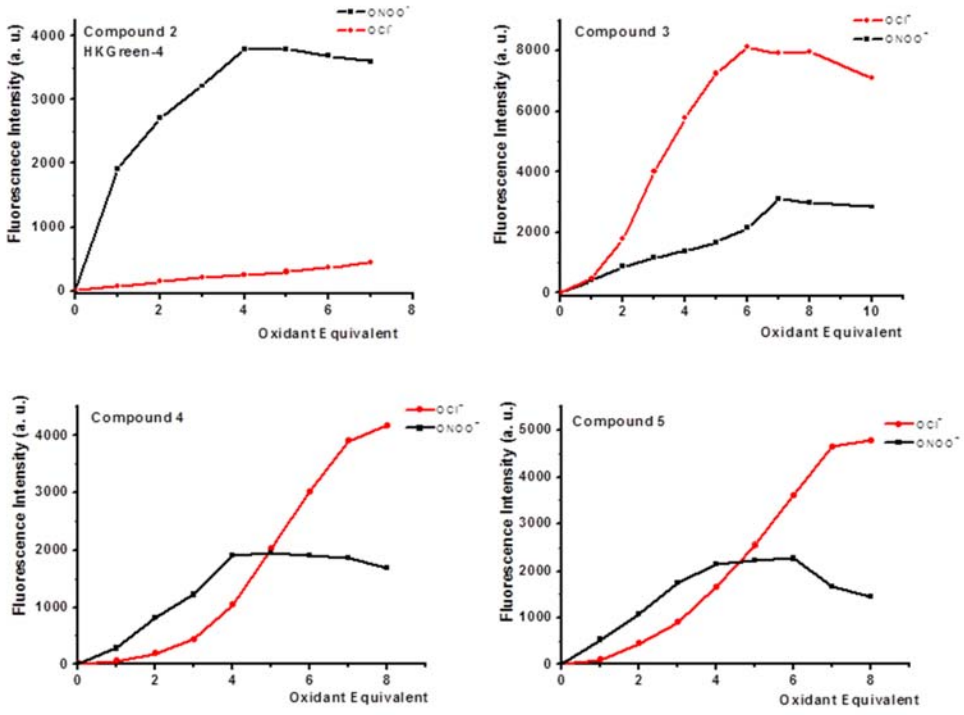
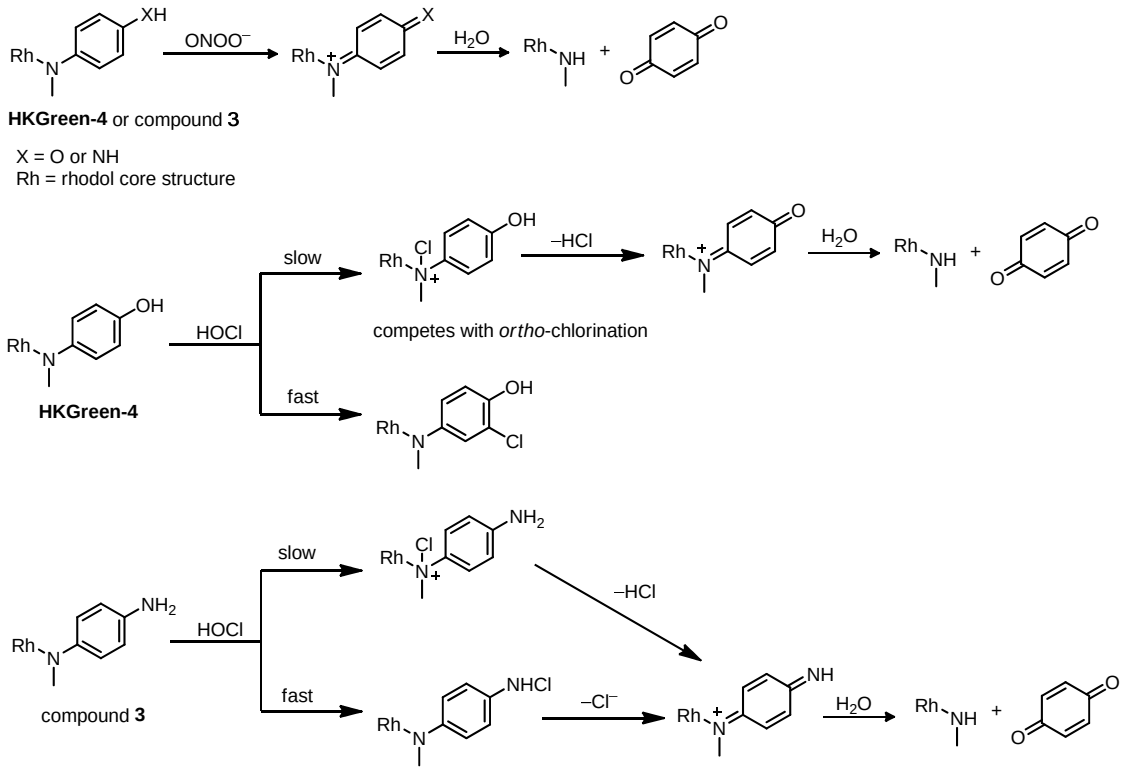


Figure S2. Fluorescence intensities of compounds **2**–**5** after treated with various amounts of hypochlorous acid and peroxynitrite. The compounds were in 0.1 M phosphate buffer (pH 7.4) at the concentration of 1 μM. The fluorescence intensities were recorded at 535 nm with excitation at 517 nm.

Scheme S6. Proposed mechanisms of reactions between **HKGreen-4**/compound **3** and $ONOO^-$/HOCl



HKGreen-4 and compound **3** are probably oxidized by peroxynitrite through two-electron oxidation to form the iminium ions, followed by water hydrolysis to afford the fluorescent product. Unlike peroxynitrite, hypochlorous acid reacts with **HKGreen-4** through *N*-chlorination of the diarylamine moiety and more preferably through chlorination at phenol *ortho* positions, inefficiently yielding the fluorescent product. By contrast, *N*-chlorination of the readily accessible amino group of compound **3** with hypochlorous acid enables the generation of the fluorescent product easily.

5. Photophysical Characterization of HKGreen-4 for Peroxynitrite Detection

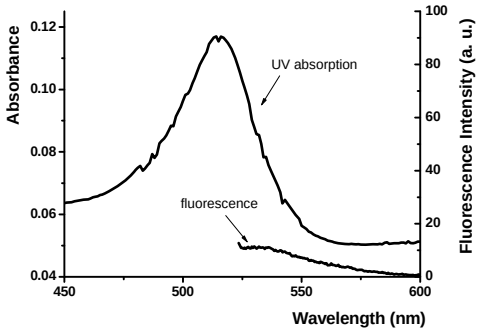


Figure S3. UV and fluorescence spectra of **HKGreen-4** (1 μM) in 0.1 M phosphate buffer at pH 7.4. The fluorescence spectrum of **HKGreen-4** was recorded with excitation at 517 nm.

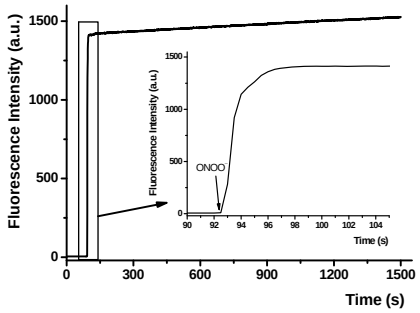


Figure S4. Time course in the detection of peroxynitrite with **HKGreen-4** monitored by fluorescence. The probe was dissolved in 0.1 M phosphate buffer (pH 7.4) at a 1 μM concentration. The fluorescence intensity was monitored with time at emission of 535 nm (excitation at 517 nm). Peroxynitrite was added into the probe solution at the time point indicated by the arrow.

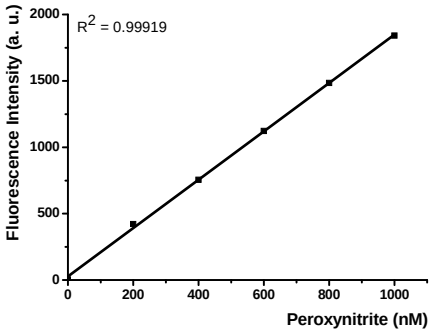


Figure S5. Linear correlation between the fluorescence emission intensity of **HKGreen-4** (1 μM in 0.1 M phosphate buffer at pH 7.4) and the concentration of peroxynitrite. The fluorescence intensity was determined at 535 nm with excitation at 517 nm.

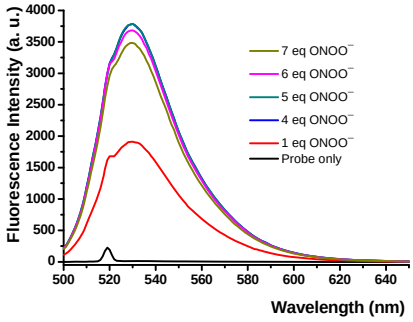


Figure S6. Fluorescence spectra of 1 μM **HKGreen-4** (0.1 M phosphate buffer at pH 7.4) in the presence of different amounts of $ONOO^-$. Spectra were acquired at 25 °C with excitation at 517 nm.

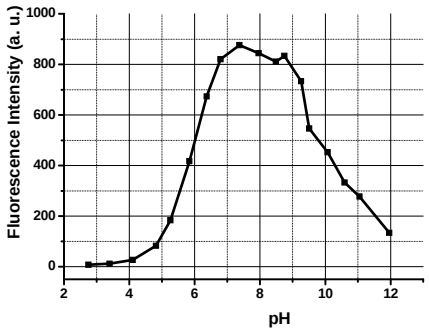


Figure S7. pH-dependent profile of **HKGreen-4** in the detection of peroxynitrite. The probe was dissolved in pH 7.4 phosphate buffer (0.1 M) at 1 μM concentration. 6 M KOH solution or

concentrated phosphoric acid was used to adjust the pH. The final concentration of added peroxynitrite was 0.5 μM. The fluorescence intensity was recorded at 535 nm with the excitation at 517 nm. The pH differences before and after peroxynitrite addition were determined to be less than 0.05. The left part of this bell-shaped curve is ascribed to the pH-dependent fluorescence of the released *N*-methylrhodol **19** and/or acid-base equilibrium of peroxynitrite, while the right part of the bell-shaped curve is probably ascribed to the deprotonation of probe phenolic OH.

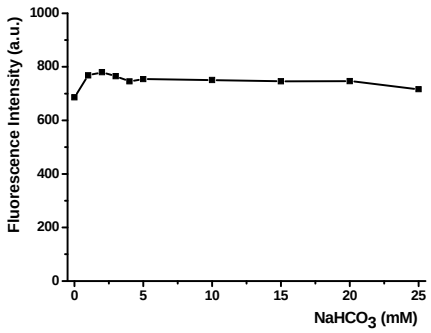


Figure S8. Effects of $NaHCO_3$ in the detection of peroxynitrite with **HKGreen-4**. The probe was dissolved in pH 7.4 phosphate buffer (0.1 M) at 1 μM concentration. Different amounts of $NaHCO_3$ were added into the probe solution to the indicated concentrations. The final concentration of added peroxynitrite was 0.5 μM.

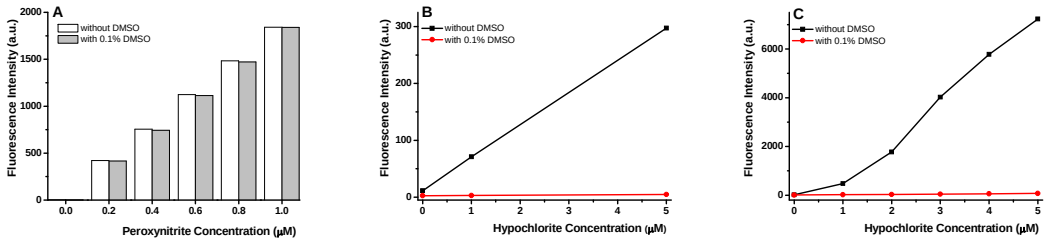
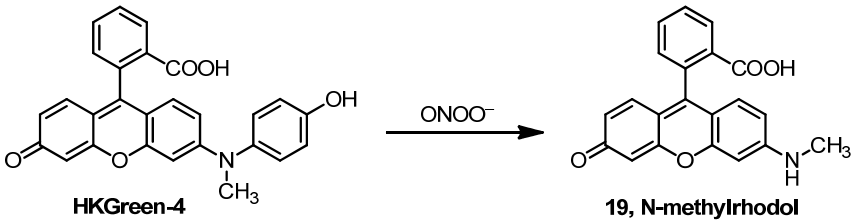


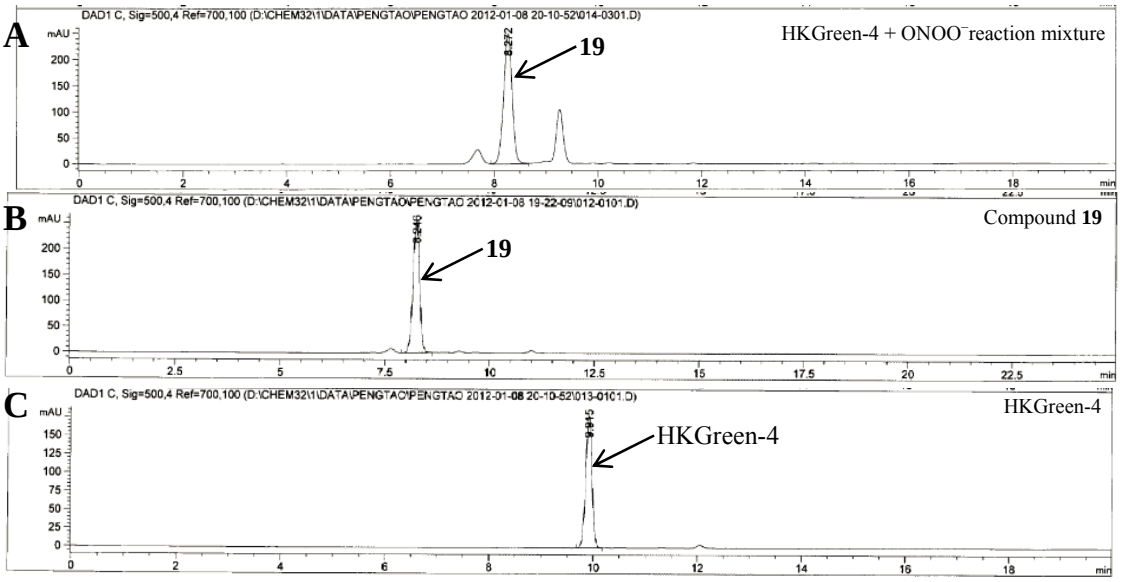
Figure S9. Effects of DMSO in the detection of peroxynitrite and hypochlorous acid with **HKGreen-4** and compound **3**. (A) Peroxynitrite detection with **HKGreen-4** (1 μM in 0.1 M phosphate buffer at pH 7.4) in the absence or presence of DMSO. (B) Responses of **HKGreen-4** toward hypochlorous acid (1 μM in 0.1 M phosphate buffer at pH 7.4) in the absence or presence of DMSO. (C) Hypochlorous acid detection with compound **3** (1 μM in 0.1 M phosphate buffer at pH 7.4) in the absence or presence of DMSO.

6. Reaction of HKGreen-4 with Peroxynitrite



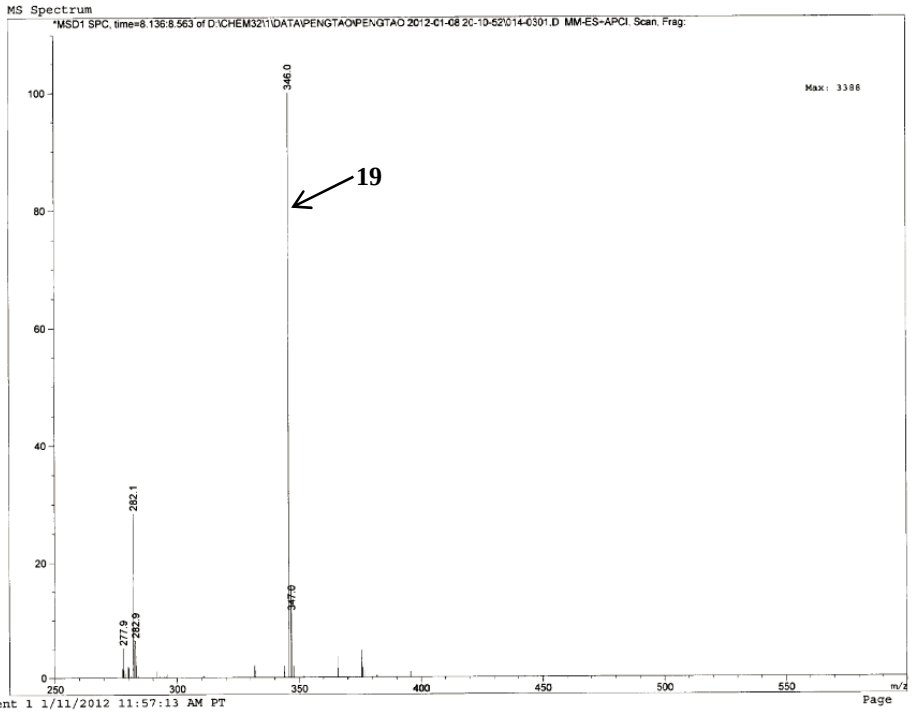
HKGreen-4 **19, N-methylrhodol**

To a solution of probe **HKGreen-4** (16 mg, 0.037 mmol) in a mixed solvent of DMF (3.5 mL) and 0.1 M phosphate buffer at pH 7.4 (350 mL) was added an alkaline solution of peroxynitrite (2.0 equiv) dropwise at rt. After stirred for half an hour, the reaction mixture was extracted with DCM/*i*-PrOH three times. The combined organic layers were dried over anhydrous Na_2SO_4, and concentrated. The resulting residue was purified by silica gel column chromatography to give the fluorescent product **19** (4 mg, ~30% yield). The residue was also analyzed by HPLC and LC-MS (Figure S10). Analytical HPLC was performed with an Agilent 1100 HPLC system. The UV detector was set at 254 nm and 500 nm. The samples were prepared as MeOH stock solutions, and were eluted from an Alltima reverse-phase C18 column (4.6 × 250 mm, 5 μm) with a linear gradient of water (containing 0.1 % TFA) and methanol (60 – 90% methanol in 10 min) at a flow rate of 1 mL/min. Samples were detected by absorbance at 500 nm, and were also identified with an Agilent 6120 Quadrupole LC/MS System coupled to the HPLC system using ESI and APCI ionization sources.



Print of window 80: MS Spectrum

D



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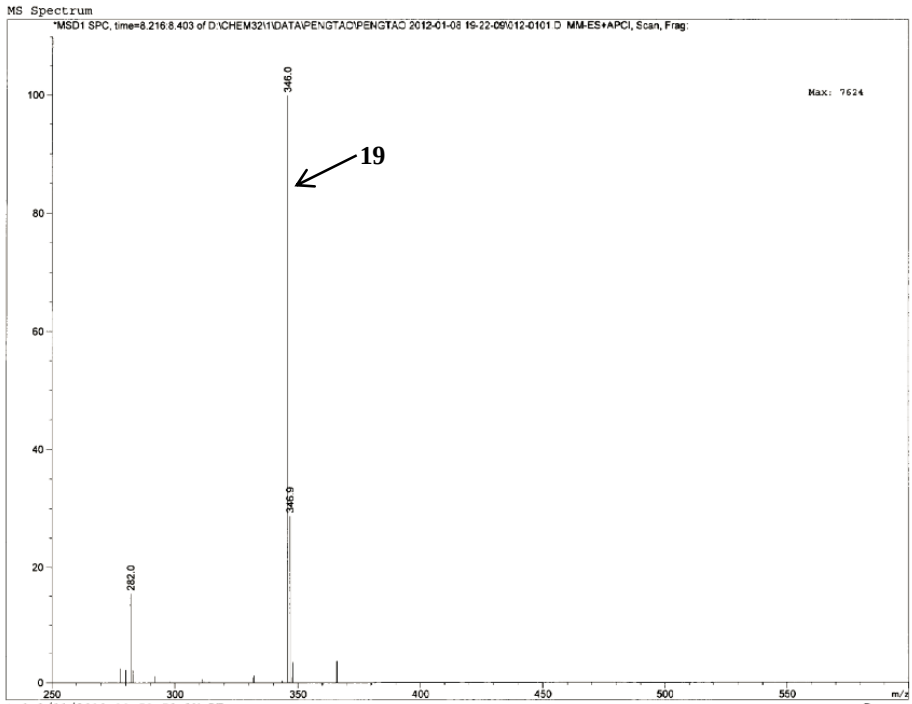
Print of window 80: MS Spectrum

E



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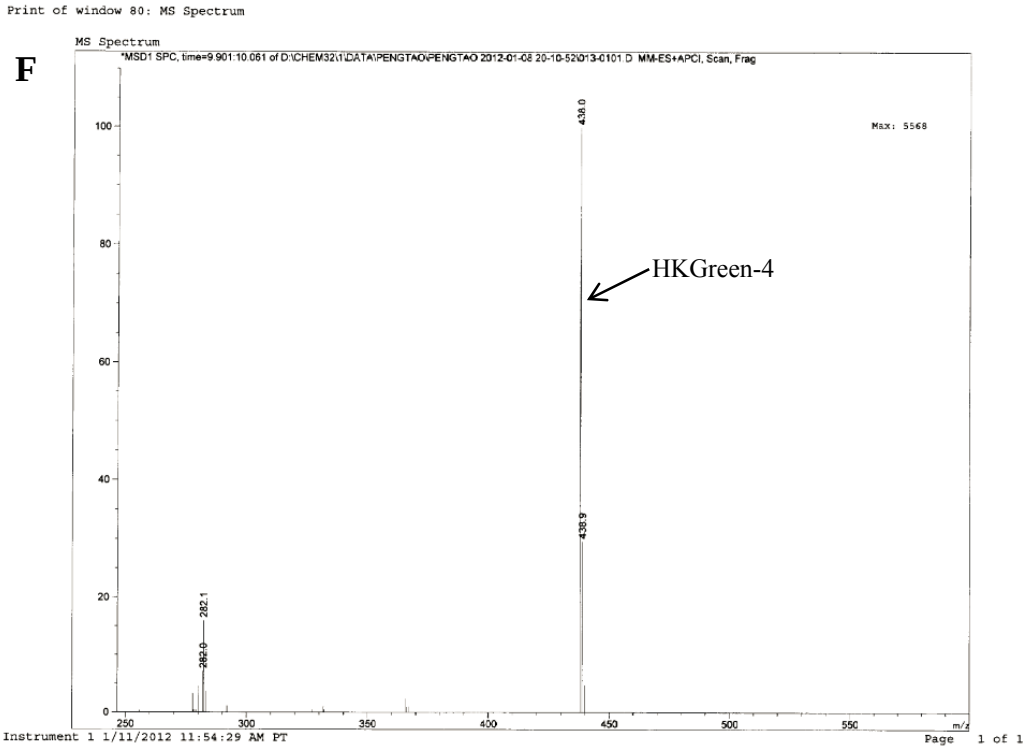

Figure S10. Detection of *N*-methylrhodol **19** as the fluorescent product in the reaction of **HKGreen-4** with peroxynitrite using HPLC and LC-MS. UV absorption was monitored at the wavelength of 500 nm with the reference set at 360 nm. (A) HPLC analysis of reaction product between **HKGreen-4** and peroxynitrite indicates the formation of **19** at the retention time of 8.2 min. (B) HPLC analysis of authentic sample of **19** indicates its retention time of 8.2 min. (C) HPLC analysis of **HKGreen-4** indicates its retention time of 9.9 min. (D) MS spectrum at the retention time of 8.2 min shown in (A) identifies the production of **19** in the reaction of **HKGreen-4** with peroxynitrite. (E) MS spectrum of the authentic sample of **19** at the retention time of 8.2 min shown in (B). (F) MS spectrum of **HKGreen-4** at the retention time of 9.9 min shown in (C).

7. Biological Assays of HKGreen-4

Cell culture

RAW264.7 mouse macrophages were acquired from ATCC and maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum (Gibco) and 1% penicillin/streptomycin, at 37 °C in 5% CO_2. The growth medium was changed every two to three days. Cells were grown to 80% confluence prior to experiment. For confocal imaging, cells were typically seeded at a density of 2×10^4 cells/mL in 35-mm confocal dish (Mat-Tek: MA, USA). BV-2 mouse microglia were acquired

as a gift from Department of Pediatrics, The University of Hong Kong, and maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum (Gibco) and 1% penicillin/streptomycin, at 37 °C in 5% CO_2. For confocal imaging, cells were typically seeded at a density of 1×10^4 cells/mL in 35-mm confocal dish (Mat-Tek: MA, USA). C17.2 mouse neural progenitor cells were acquired as a gift from School of Chinese Medicine, The University of Hong Kong, and maintained in DMEM (high-glucose; 4 mM L-glutamine; sodium pyruvate) supplemented with 10% heat-inactivated fetal bovine serum (Gibco) and 1% penicillin/streptomycin, at 37 °C in 5% CO_2. CHO (Chinese hamster ovarian) cells were acquired as a gift from Department of Chemistry, The University of Hong Kong, and maintained in MEM (with GlutaMAX; Gibco) supplemented with 10% heat-inactivated fetal bovine serum (Gibco) and 1% penicillin/streptomycin, at 37 °C in 5% CO_2. To generate primary mouse bone marrow-derived macrophages, bone marrow cells were collected from femur and tibia, and treated with RBC lysis buffer to deplete red blood cells. Resultant bone marrow progenitor cells were then differentiated into macrophages with 20 ng/mL M-CSF (macrophage colony-stimulating factor) for 5 days.

MTT assay

MTT assay was utilized to investigate the cytotoxicity of **HKGreen-4A**. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) is a yellow tetrazolium salt and can be reduced to formazan crystals, which are insoluble in aqueous solutions, by active mitochondria in living cells. The resulting intracellular purple formazan can be dissolved in acidified sodium dodecyl sulfate (SDS), and therefore quantified by measuring absorption of the solution. To build a standard curve or a growth curve, the cells were seeded in a 96-well plate and incubated with 200 μL of culture media for 2 h. Then different amounts of probes were added to the wells for further incubation of indicated times. For the standard curve, no probe was added. The culture media was then removed. Portions of 100 μL MTT solution (0.5 mg in 1 mL of Hanks' balanced salt solution) were added to the wells and the cells were further incubated at 37 °C for 2 h. Solubilization solutions (100 μL) were then added and incubated with the cells overnight. The absorption of each well was measured at a wavelength of 570 nm with a multiwell scanning spectrophotometer (ELISA reader). The cells viability was calculated according to the following equation:

$$\text{Cell viability (\%)} = 100 \times A_{\text{with probe}} / A_{\text{control}}.$$

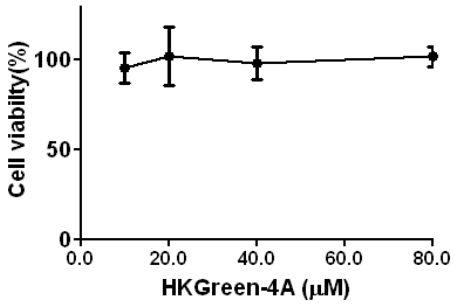

Figure S11. Cytotoxicity of **HKGreen-4A** in cultured RAW 264.7 macrophage cells. Cells were incubated with the probes at corresponding concentrations for 24 h. Cell viability was measured by MTT assay and the results are reported as percentage relative to untreated cells (mean ± SD).

Bacterial culture and moi determination

To generate a standard curve for estimating moi (multiplicity of infection), serial plating of bacterial culture diluents of known $O.D._{600nm}$ was performed. Briefly, antibiotic-free LB (Luria broth) medium (4 mL) was inoculated with E. coli (strain JM109) recovered from liquid nitrogen stock (30% sterile glycerol), for overnight incubation at 37°C with rigorous shaking at 220 rpm (Centrifuge 5804; Eppendorf: Hamburg, Germany). Bacteria were allowed to grow to log phase ($O.D._{600nm}$ ~0.6 – 0.8) and were harvested by centrifugation at 1,000 rpm (Centrifuge 5804; Eppendorf: Hamburg, Germany) for 10 min. Cell pellet was resuspended in 1 mL sterile HBSS (Hank's balanced salt solution) and diluted to a suitable range of O.D. (optic density) at 600 nm: 0.1 to 1. After $O.D._{600nm}$ determination, resuspended bacteria were further serially diluted (successively by a dilution factor of 10) in 2-mL Eppendorf tubes, for the following range of dilution factors: 10×, 10^2×, 10^3×, 10^4×, 10^5×, 10^6×. For bacteria of different $O.D._{600nm}$, a 100-μL inoculum (at 10^5× and 10^6×) was aseptically plated unto antibiotic-free LB agar plates in triplicates, and incubated for 2 days at 37°C. Resultant colonies on the triplicate plates were counted to obtain a mean count corresponding to CFU counts (number of colony-forming units). CFU counts were plotted as a dependent variable against corresponding $O.D._{600nm}$ as an independent variable to obtain a standard curve. Prior to bacterial infection experiment, overnight liquid culture of E. coli was grown to log phase in LB at 37°C. After harvest by centrifugation, cell pellet was washed 1× with HBSS, resuspended and diluted with HBSS to concentrations suitable for O.D. measurement (usually about $O.D._{600nm}$ = 0.8 – 2). Based on the measured $O.D._{600nm}$, an estimated CFU count was obtained from the pre-determined standard curve above. Bacteria were then further diluted with HBSS to a desired moi for infection of macrophages.

Preparation of heat killed bacteria

Heat-killed *E. coli* only was used for confocal fluorescence imaging, in compliance with local ethics regulations for microscopy in biomedical research. *E. coli* was grown to log phase and harvested by centrifugation. After determination of $O.D._{600nm}$ and dilution with HBSS to suitable moi, *E. coli* suspension in 2-mL Eppendorf tubes was incubated in a Stuart block heater (Bibby Scientific: Staffordshire, UK) at 90°C for 40 min. Immediately before treatment, bacteria were thoroughly resuspended by rigorous vortex and pipetting.

Confocal fluorescence imaging in live cells

For induction of endogenous $O_2^{\bullet-}$ and $ONOO^-$ formation, RAW264.7 cells were seeded into 35-mm glass-bottom culture dishes (Mat-Tek: Ashland MA, USA) in DMEM medium (normal glucose) supplemented with 10% FBS (fetal bovine serum), 1% P/S (penicillin/streptomycin; Gibco-Invitrogen) in a seeding volume of 2 mL per well, at a density of 2×10^4 cells/mL. After attachment and overnight culture at 37°C, old culture medium was discarded, followed by washing with sterile HBSS to remove residual FBS and medium dye. Heat-killed *E. coli* (moi = 100) suspension was diluted with treatment medium (DMEM without phenol red, supplemented with 3% FBS, 0.6 mM L-arginine, 0.01% chloramphenicol) and subsequently added to RAW264.7 cells in a treatment volume of 1.5 mL per dish. Prior to desired time points of imaging (usually, 30 min before t = 14 h post-infection), bacteria-infected or drug-treated RAW264.7 cells were loaded with appropriate fluorescent dyes and further incubated at 37°C for 30 min before image acquisition. Briefly, MitoSOX Red (2.5 μM), **HKGreen-4A** (10 μM) and the nuclear DNA dye Hoechst 33342 (75 ng/mL; Invitrogen Molecular Probes) were added to 1 mL recovered treatment medium, mixed and immediately returned to the culture dish. At the end of 30-min probe incubation, the culture dish was mounted onto a live cell support module (AxioVision; Zeiss: Jena, Germany) maintained at 37°C and 5% CO_2. Imaging was performed with LSM Meta 510 (Zeiss: Jena, Germany). Z-stack imaging was conducted with an oil lens by acquiring 5 consecutive photosections (0.37 μm per section) at 63×4 magnification about the equatorial region of host cells (where diameters of the nuclei are generally largest) with the following imaging parameters: MitoSOX Red (λ_{ex} = 543 nm; λ_{em} = 565-615 nm band-pass; laser intensity = 26%), **HKGreen-4A** (λ_{ex} = 488 nm; λ_{em} = 500-550 nm band-pass; laser intensity = 26%), and Hoechst 33342 (two-photon mode; λ_{ex} = 790 nm; λ_{em} = 435-485 nm band-pass; laser intensity = 3%). Photosections from respective channels were collapsed into a single merged image and exported in appropriate image format (TIF or JPG). For imaging of cell morphology and probe distribution, the mitochondrial organelle dye MitoTracker Red (100 nM; Invitrogen Molecular Probes) was used in place of MitoSOX

Red.

Confocal fluorescence imaging in live tissues

For detection of $ONOO^-$ under atherosclerotic conditions, apolipoprotein E knockout ($ApoE^{-/-}$) mice were chosen as an atherosclerosis model to compare with wild-type C57BL/6 mice, which served as a reference for basal $ONOO^-$ formation in healthy animals. Each group (n = 6 males) was fed on normal chow diet for 20 weeks. Atherosclerotic plaques and other symptoms had been confirmed in the $ApoE^{-/-}$ mice by conventional histological methods (oil red O staining) and microscopic inspection. Mice were fasted overnight before tissue sampling. 10% pentobarbital (0.1 mL/g) was injected as an anesthetic during sacrifice. Mouse abdomen and chest were surgically opened to reveal the heart. A small aperture was made by cutting at the right atrium. Initial perfusion (5 min) was performed with HBSS (Hank's balanced salt solution; Gibco) to remove blood. **HKGreen-4A** (2 μM) was next added to the perfusion buffer (total 10 mL HBSS, supplemented with 0.6 mM L-arginine for sustaining $^{\bullet}NO$ production) for tissue staining during perfusion (10 min). The heart was then excised and bathed in the perfusion buffer with **HKGreen-4A** (2 μM) and 0.6 mM L-arginine, for another 20 min to allow sufficient incubation. To avoid nonselective ROS induction and heterogeneous tissue decay, staining was terminated by fixing with 4% PFA (paraformaldehyde) in cold PBS, followed by quenching with 100 mM glycine and wash with PBS for desalting. The heart tissues were embedded in OCT (optimal cutting temperature) compound and stored at −20°C. The tissues were then cryosectioned at an interval of 10 μm. Regions of interest, the smooth muscles of mouse aortic root, were selected and mounted onto a glass slide (Thermo Scientific). Image acquisition was performed with LSM Meta 510 (Zeiss: Jena, Germany). Z-stack imaging was conducted with an oil lens by acquiring 5 or 20 consecutive photosections (0.37 μm per section) at 63×1 magnification with the following imaging parameters for **HKGreen-4A**: two-photon mode; λ_{ex} = 730 nm; λ_{em} = 500-550 nm band-pass; laser intensity = 4%. Photosections from were collapsed into a single merged image and exported in appropriate image format (TIF or JPG) before image processing and analysis.

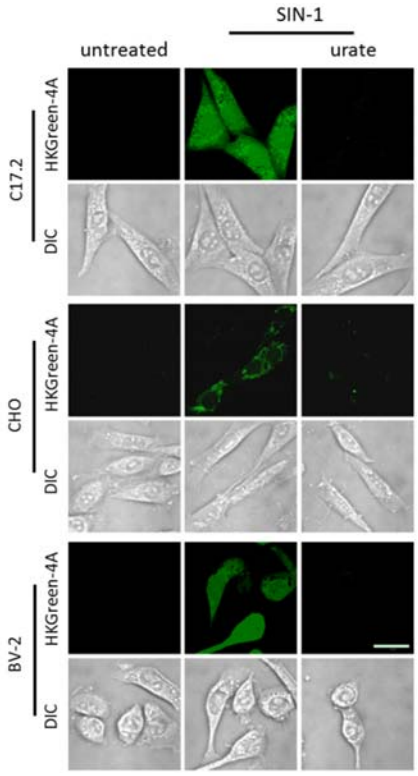

Figure S12. Imaging of exogenous $ONOO^-$ with **HKGreen-4A** in different types of live cells. C17.2 mouse neural progenitor cells, CHO (Chinese hamster ovarian) cells, or BV-2 mouse microglia were co-incubated with **HKGreen-4A** (10 μM) and SIN-1 (50 μM) as $ONOO^-$ donor for 1 h, followed by confocal imaging. Sodium urate (100 μM) was used as an $ONOO^-$ scavenger. "DIC" represents bright field image. Scale bar represents 20 μm.

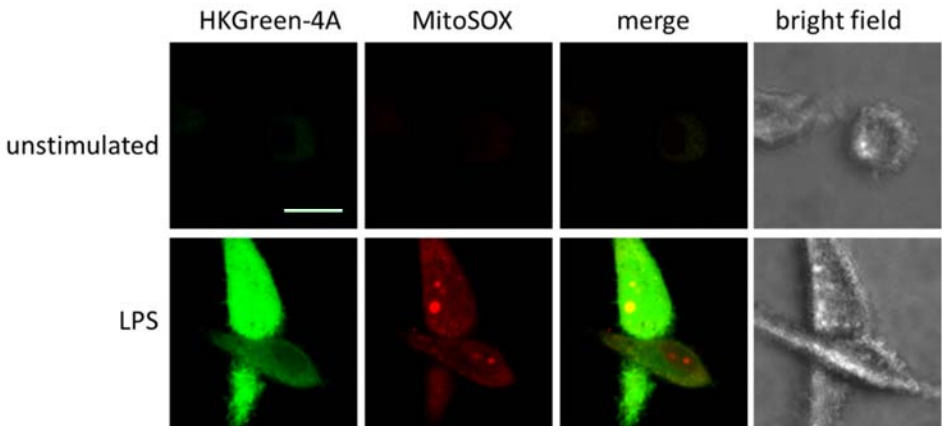

Figure S13. Imaging of endogenous $ONOO^-$ with **HKGreen-4A** in primary macrophages. Mouse bone marrow progenitor cells were differentiated with M-CSF (20 ng/mL; 5 days) into macrophages, which were then stimulated with LPS (*E. coli* K12; 1 μg/mL) for 18 h. Cells were co-incubated with **HKGreen-4A** (10 μM) and MitoSOX Red ($O_2^{\bullet-}$ probe; 2.5 μM) for 30 min, before confocal imaging. Scale bar represents 10 μm.

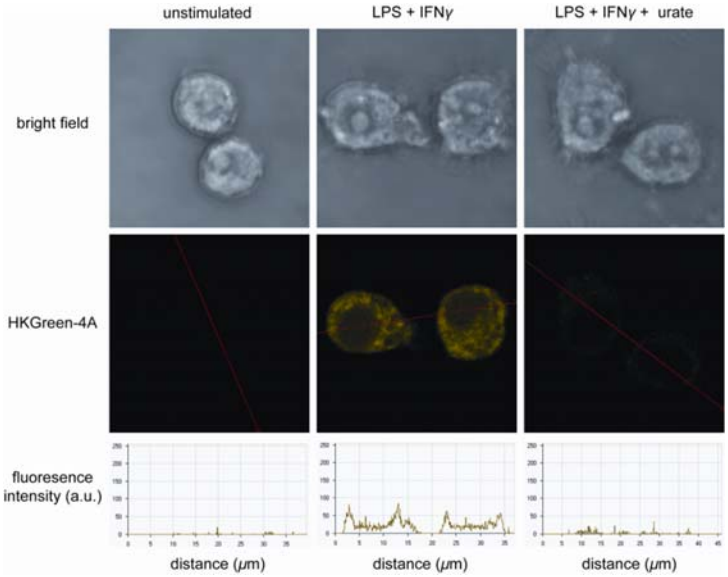

Figure S14. Two-photon confocal imaging of endogenous $ONOO^-$ with **HKGreen-4A** in RAW264.7 mouse macrophages. Cells were stimulated with LPS (*Salmonella t.*; 1 μg/mL) plus IFN-γ (mouse; 100 ng/mL) for 14 h. Cells were incubated with **HKGreen-4A** (10 μM) for 30 min, before confocal imaging (two-photon mode; λ_{ex} = 730 nm, λ_{em} = 500-550 nm band-pass; laser intensity = 4%). Sodium urate (100 μM) was used to scavenge $ONOO^-$. Profiles of fluorescence intensity across selected region of

interest were generated with LSM Meta 510 image analysis software.

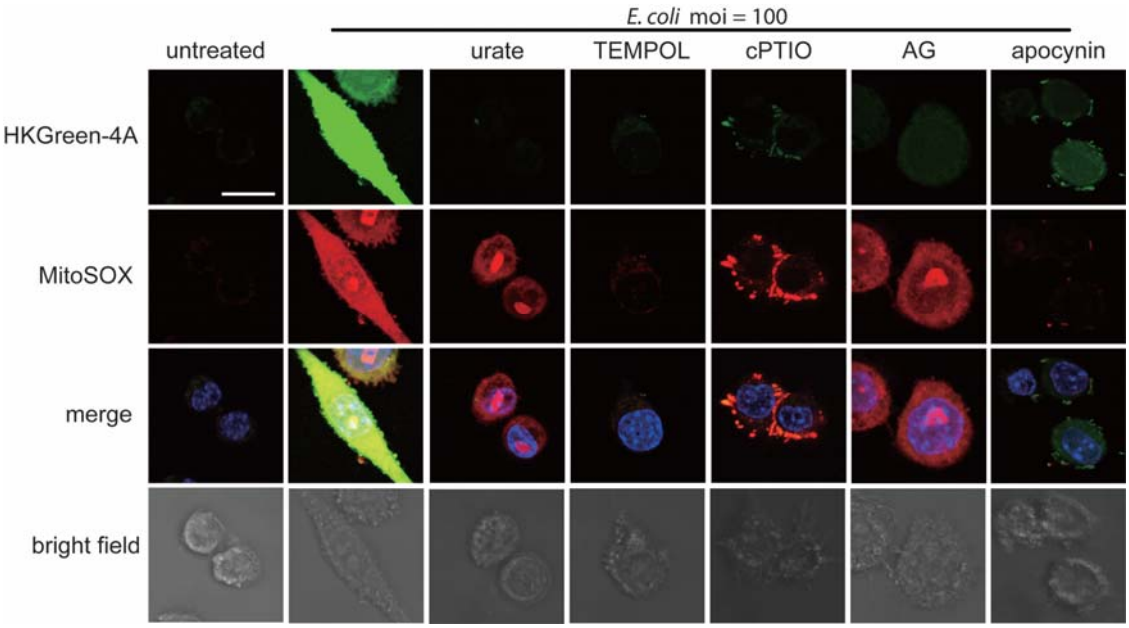

Figure S15. Validation of enzymatic pathways involved in endogenous $ONOO^-$ formation in *E. coli*-challenged RAW264.7 macrophages. Cells were treated with heat-killed *E. coli* (moi = 100) for 14 h, and then stained with **HKGreen-4A** (10 μM), MitoSOX (2.5 μM), and Hoechst 33342 (75 ng/mL) in the presence or absence of urate ($ONOO^-$ scavenger; 100 μM), TEMPOL ($O_2^{\bullet -}$ scavenger; 300 μM), or cPTIO ($^{\bullet}NO$ scavenger; 125 μM) for 30 min, before confocal imaging. Aminoguanidine (AG; 300 μM) and apocynin (500 μM), which inhibit NOS and NOX, respectively, were added to cells during stimulation. "Merge" represents overlays of all fluorescence channels including Hoechst. Scale bar represents 10 μm.

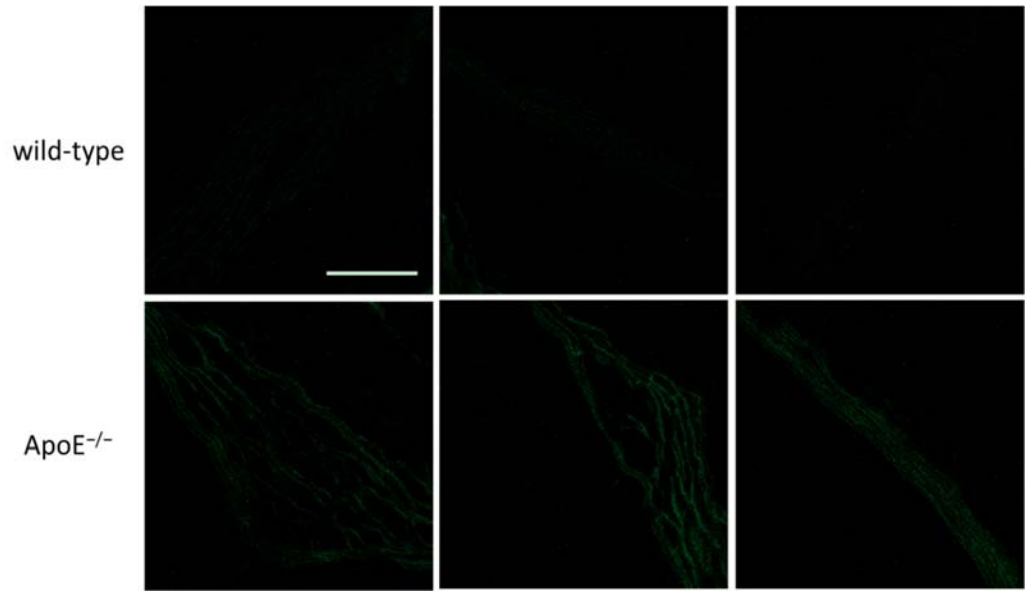

Figure S16. Representative imaging of endogenous $ONOO^-$ in single photosections of smooth muscles in mouse aortic root in a mouse model of atherosclerosis. Living smooth muscles of the mouse aortic root were stained with **HKGreen-4A** (2 μM) for 30 min by perfusion, fixed, cryosectioned and mounted for two-photon confocal imaging. Six animals were used in each group. Scale bar represents 50 μm.

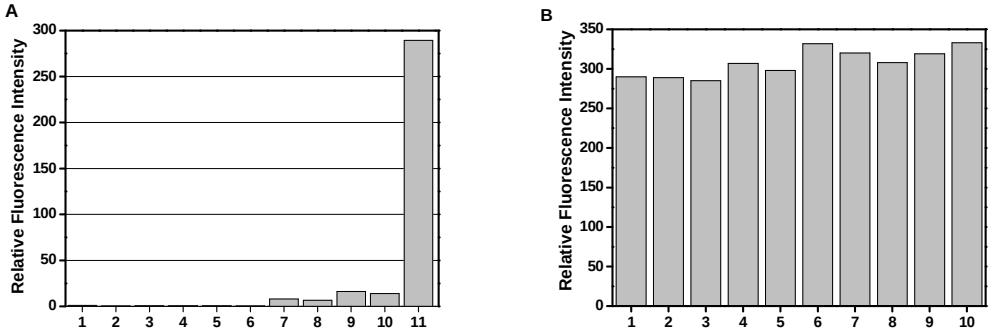

Figure S17. (A) Fluorescence increases of **HKGreen-4** (1 μM) towards various ROS and RNS. (1) probe only; (2) 10 equiv $^1O_2$; (3) 10 equiv $^\bullet NO$; (4) 10 equiv $ROO^\bullet$; (5) 10 equiv $O_2^{\bullet-}$; (6) 10 equiv H_2O_2; (7) 10 equiv NO_2; (8) 10 equiv $CO_3^{\bullet-}$; (9) 1 equiv $^\bullet OH$; (10) 1 equiv HOCl; (11) 1 equiv $ONOO^-$. (B) Fluorescence increases of **HKGreen-4** (1 μM) towards $ONOO^-$ (1 equiv) in the presence of various ROS and RNS. (1) 1 equiv $ONOO^-$ only; (2) 10 equiv $ROO^\bullet$ plus 1 equiv $ONOO^-$; (3) 10 equiv $^1O_2$ plus 1 equiv $ONOO^-$; (4) 10 equiv H_2O_2 plus 1 equiv $ONOO^-$; (5) 10 equiv $^\bullet NO$ plus 1

equiv $ONOO^-$; (6) 10 equiv $O_2^{\bullet-}$ plus 1 equiv $ONOO^-$; (7) 10 equiv NO_2 plus 1 equiv $ONOO^-$; (8) 10 equiv $CO_3^{\bullet-}$ plus 1 equiv $ONOO^-$; (9) 1 equiv $^{\bullet}OH$ plus 1 equiv $ONOO^-$; (10) 1 equiv HOCl plus 1 equiv $ONOO^-$.

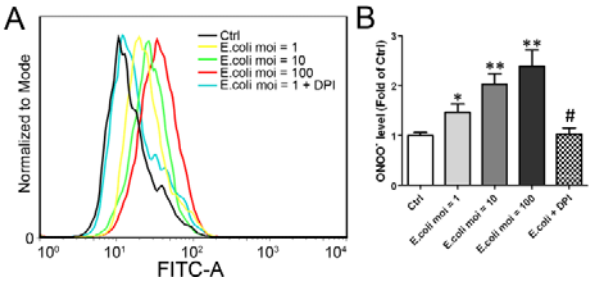

Figure S18. Flow cytometry analysis of **HKGreen-4**-loaded live RAW 264.7 macrophages in response to stimulation with heat-killed *E. coli*. Cells were challenged with heat-killed *E. coli* (moi = 1, 10, 100) for 15 h and incubated with **HKGreen-4A** (20 μM) at 37°C for 30 min. Cells were then harvested, resuspended in PBS and analyzed by flow cytometry (Becton-Dickinson LSRII, Franklin Lakes, NJ, USA). (A) Representative histogram of $ONOO^-$ generation in macrophages upon stimulation with heat-killed *E. coli* (moi = 1, 10, 100) in the presence or absence of DPI (50 nM). (B) Statistic analysis of traces in (A), data presented as mean relative fluorescence intensity ± SD (n = 3). *p < 0.05 and **p < 0.01, versus control group; #p < 0.05, versus *E. coli* (moi = 1) group.

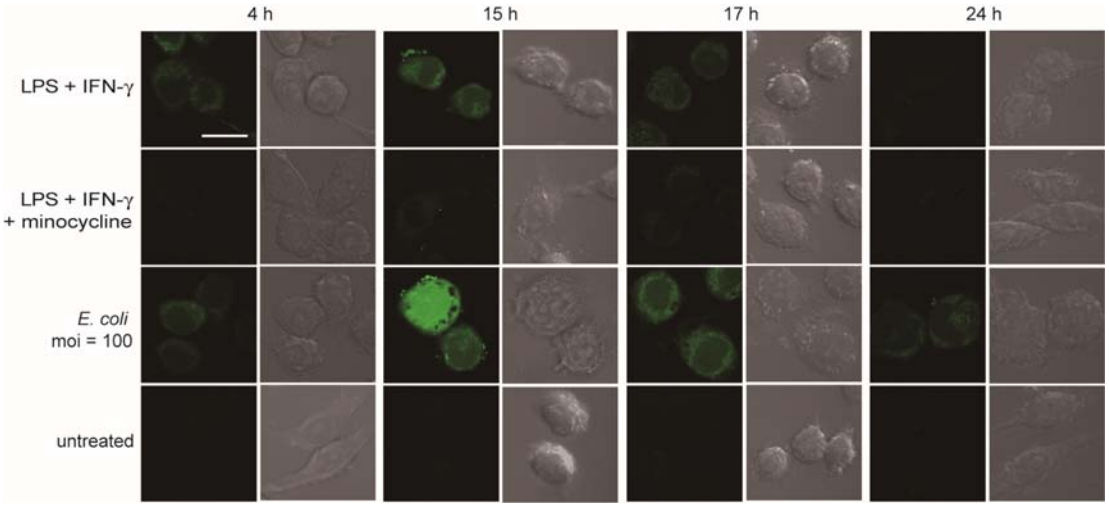

Figure S19. Complete figure of Figure 3A including bright field images.

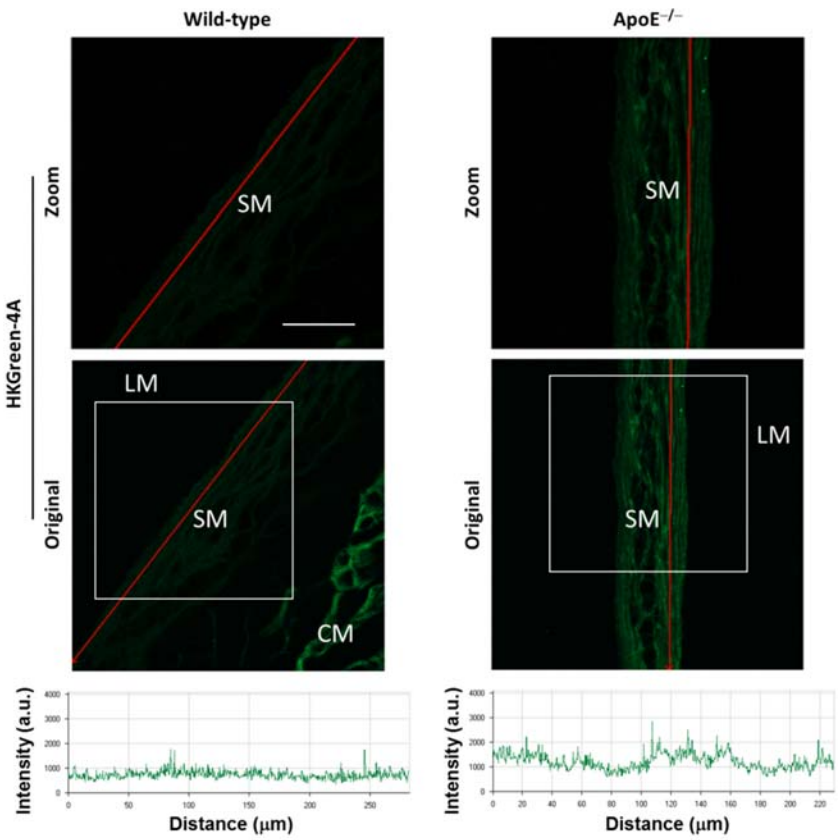

Figure S20. Original uncropped images of Figure 5. SM, CM, and LM refer to smooth muscles, cardiac muscles, and lumen, respectively. The white rectangles indicate the cropped regions shown in Figure 5. Scale bar represents 50 μm.

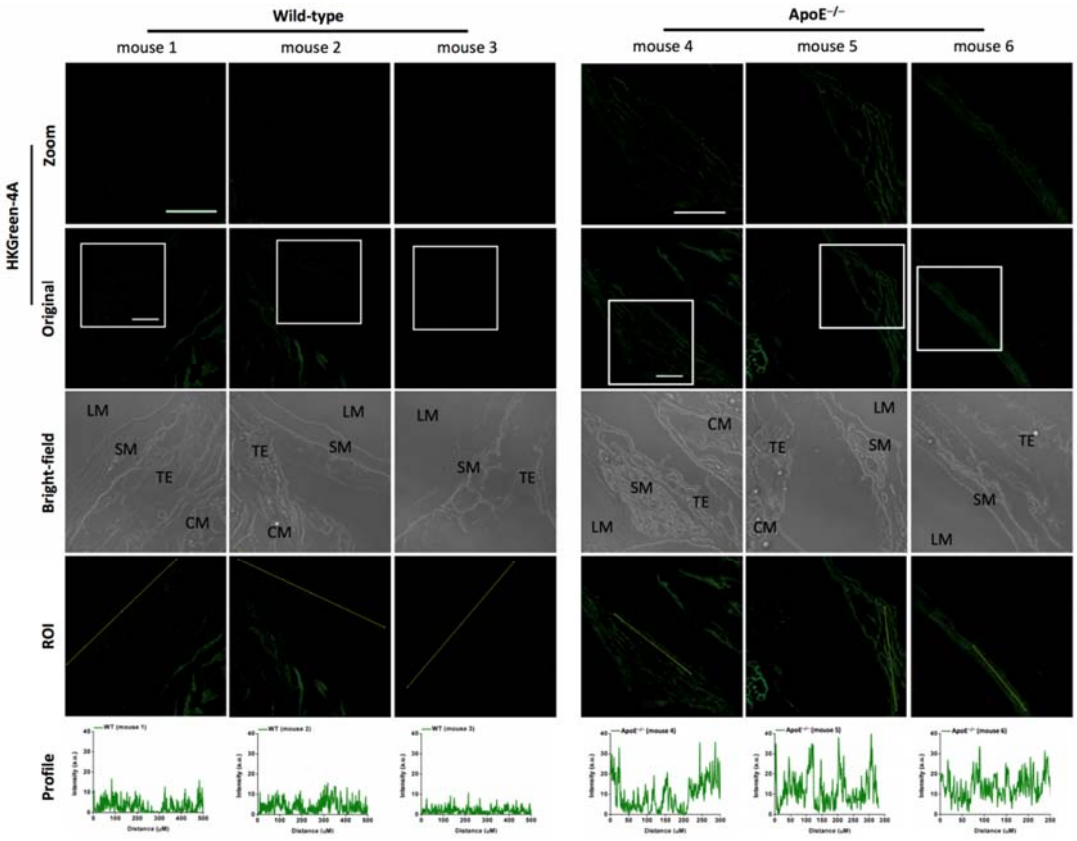

Figure S21. Original uncropped images of Figure S16. SM, CM, LM, and TE refer to smooth muscles, cardiac muscles, lumen, and tunica externa, respectively. The white rectangles indicate the cropped regions shown in Figure S16. Profiles of fluorescence intensity of regions of interest (ROI) are shown as indicated. Scale bar represents 50 μm.

8. NMR spectra

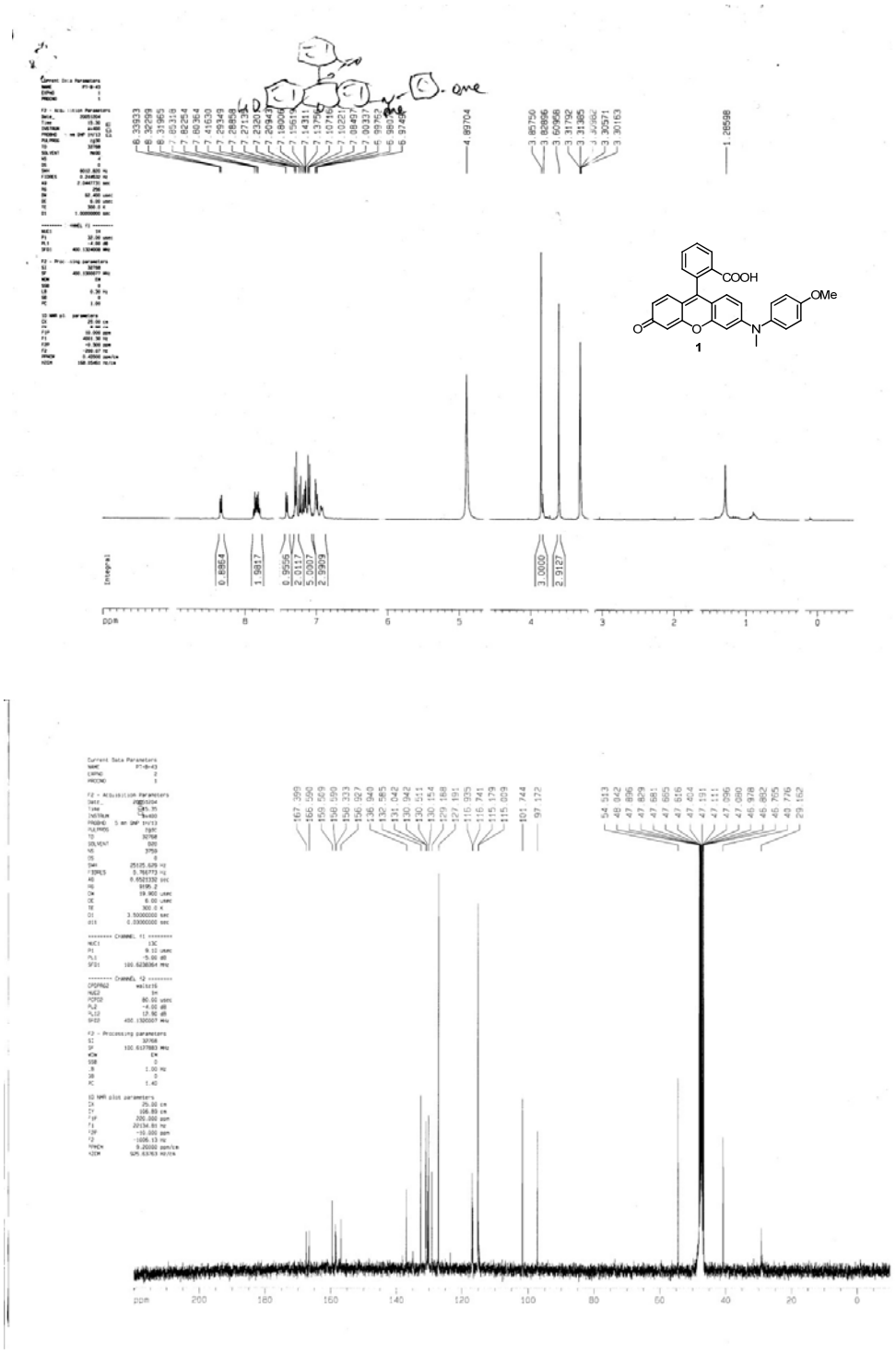

Figure S22. ^{1}H and ^{13}C NMR spectra of compound **1**.

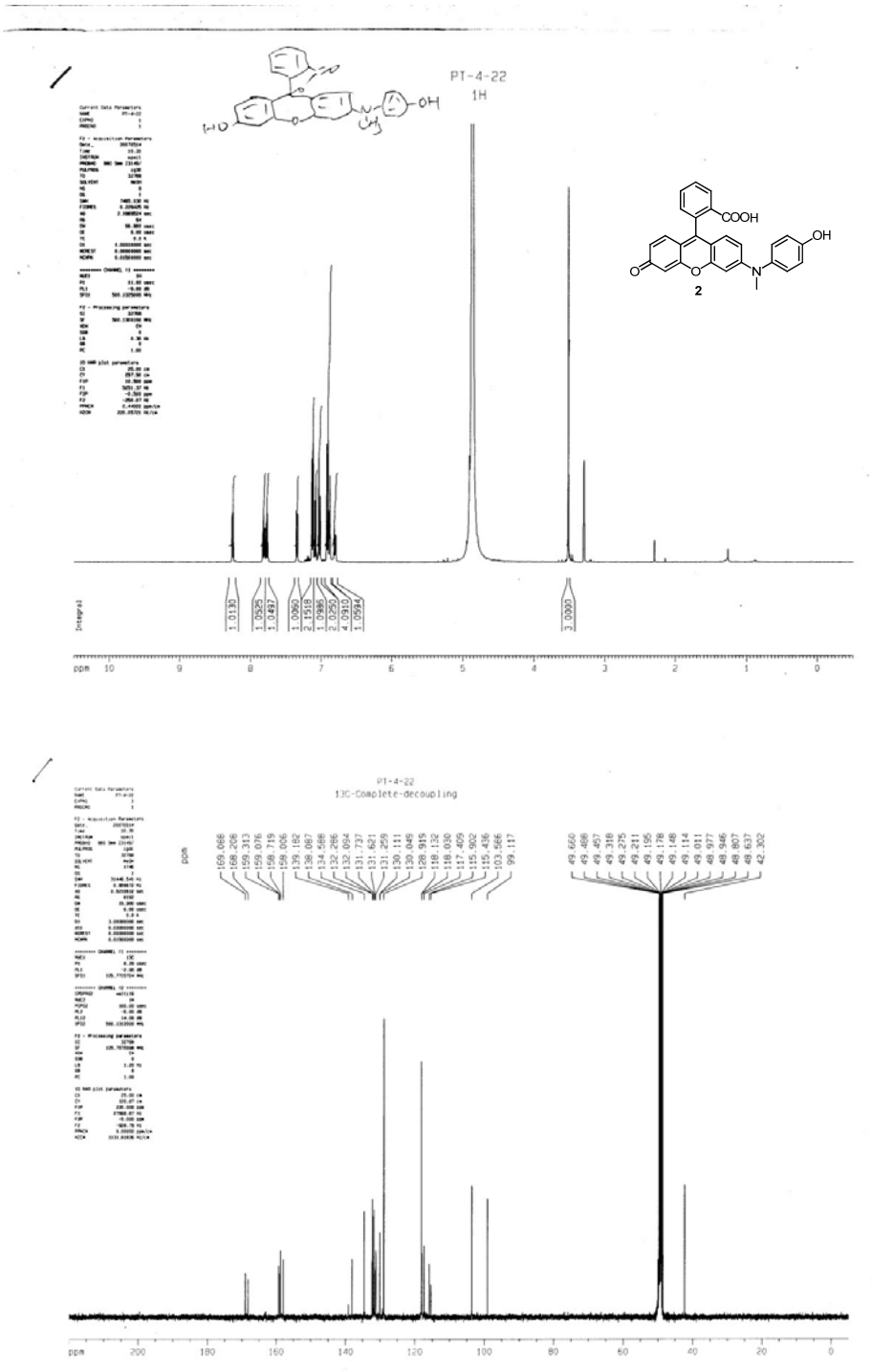

Figure S23. ^{1}H and ^{13}C NMR spectra of compound **2**.

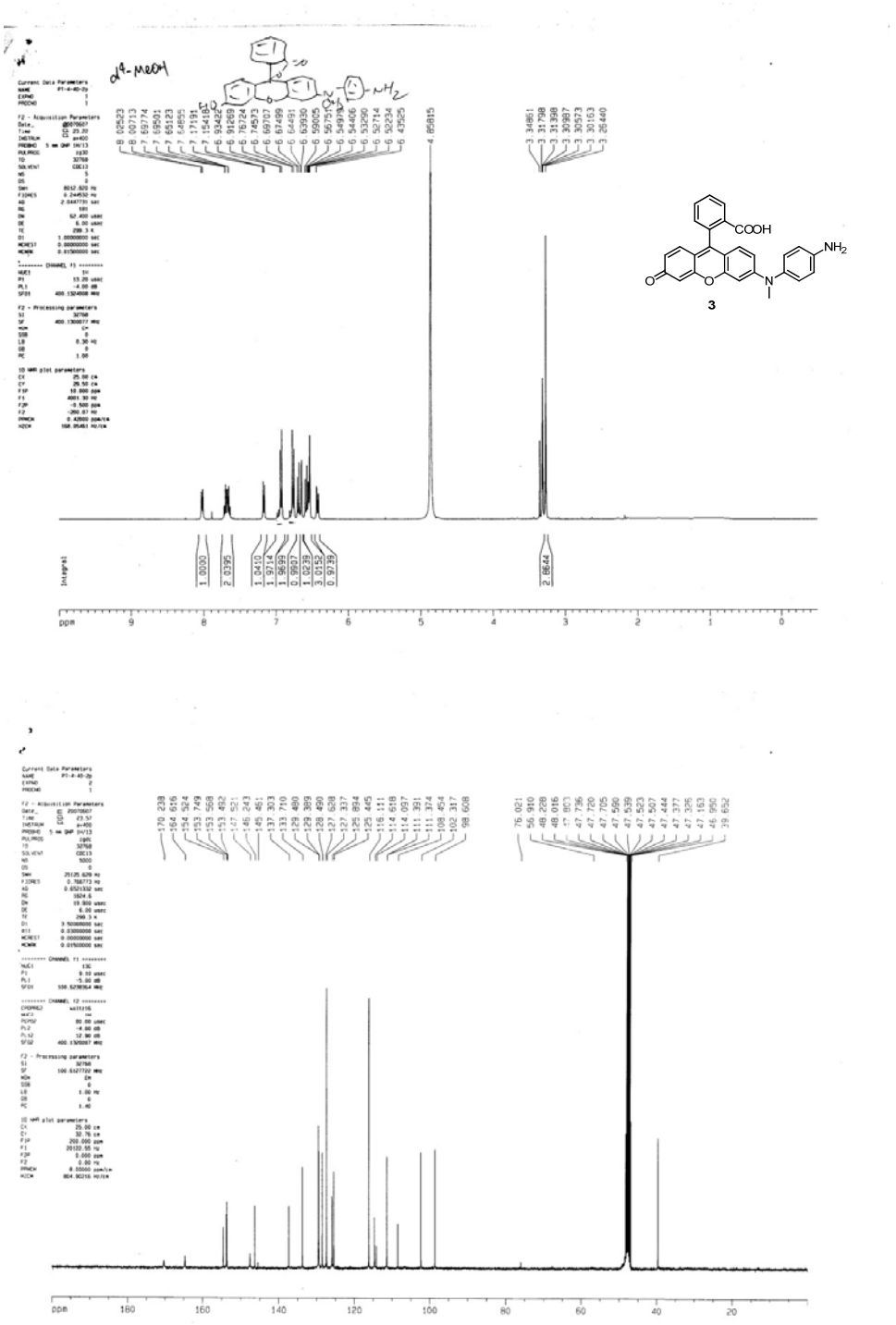

Figure S24. ^{1}H and ^{13}C NMR spectra of compound **3**.

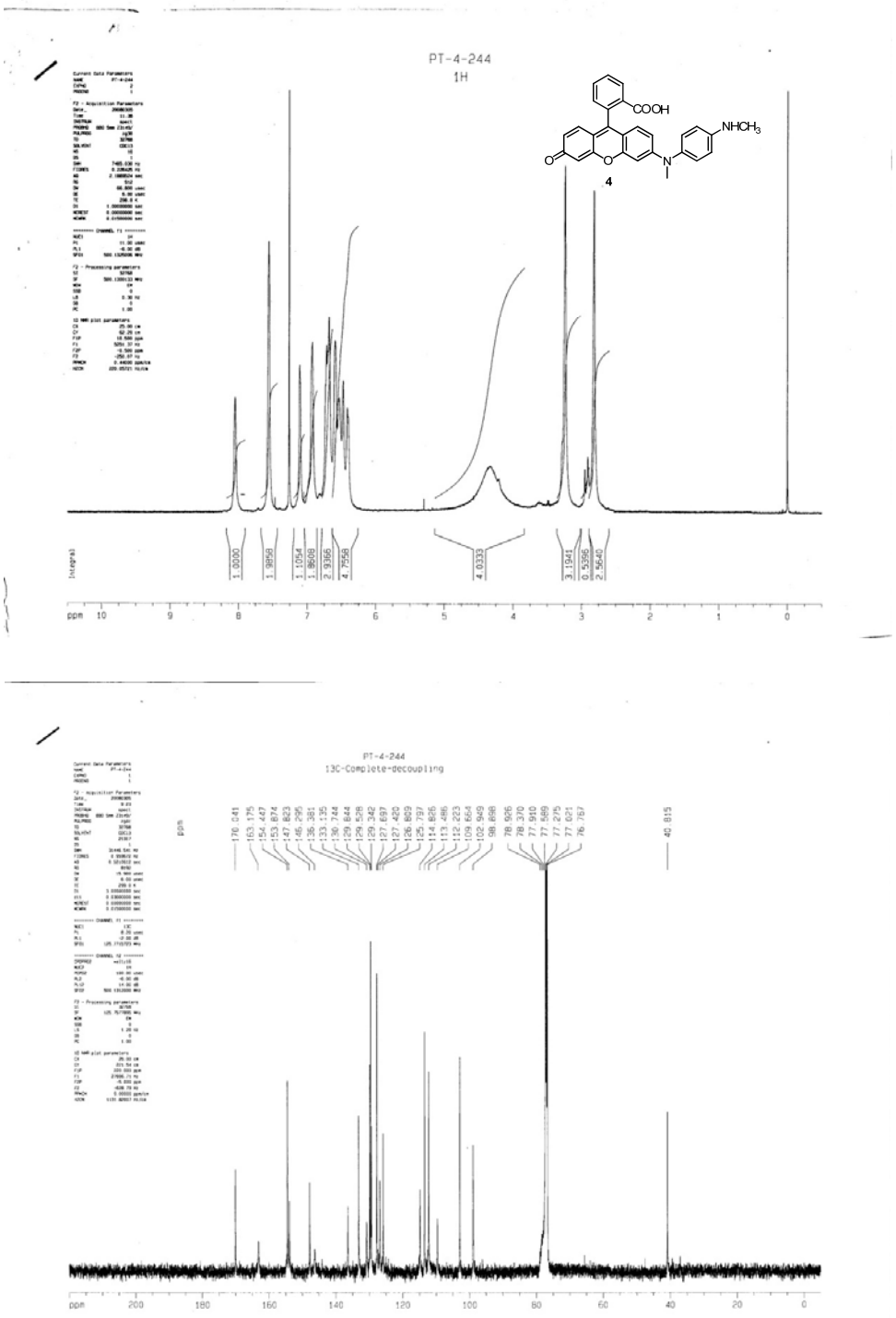

Figure S25. ^{1}H and ^{13}C NMR spectra of compound **4**.

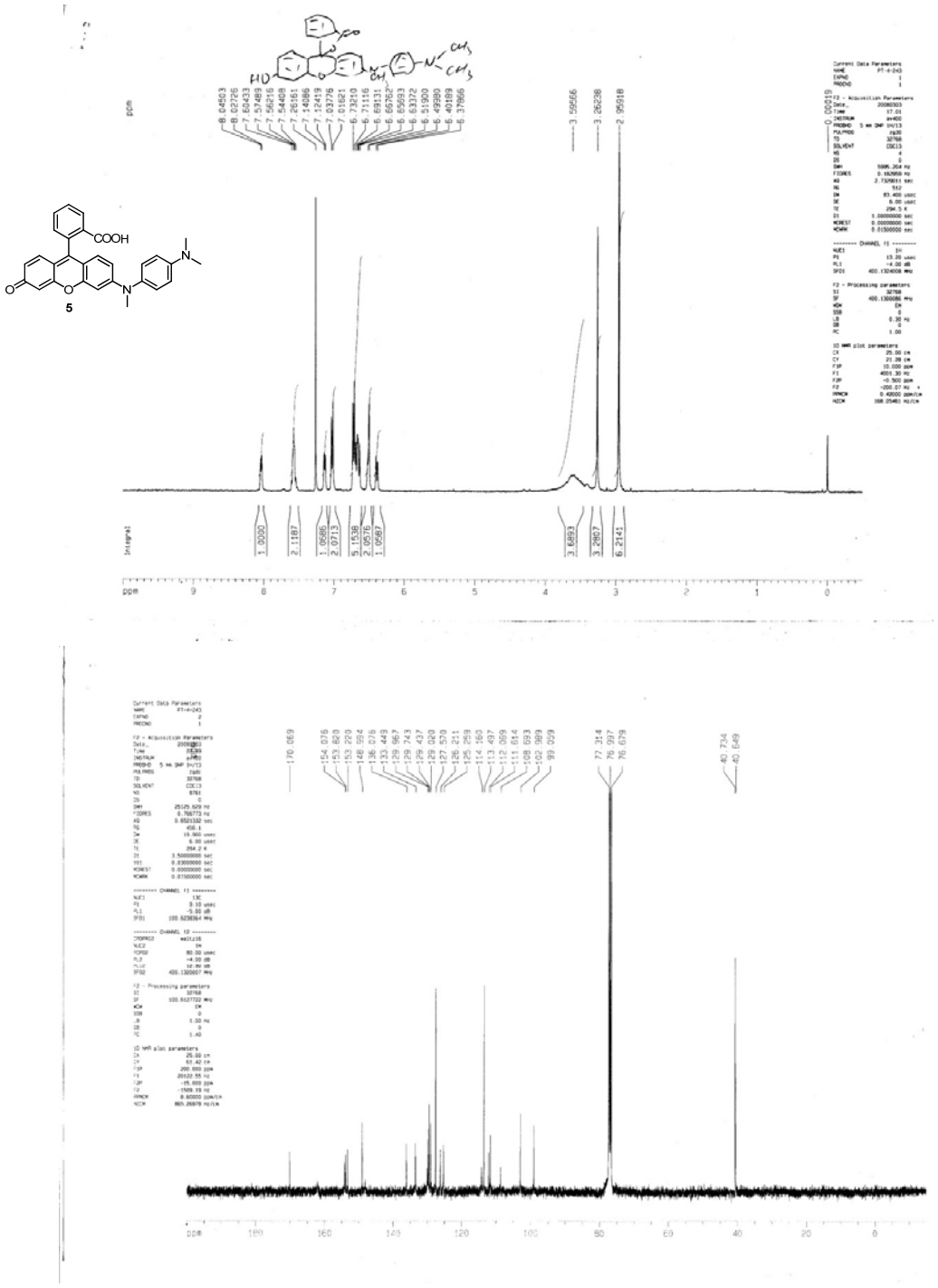

Figure S26. ^{1}H and ^{13}C NMR spectra of compound **5**.

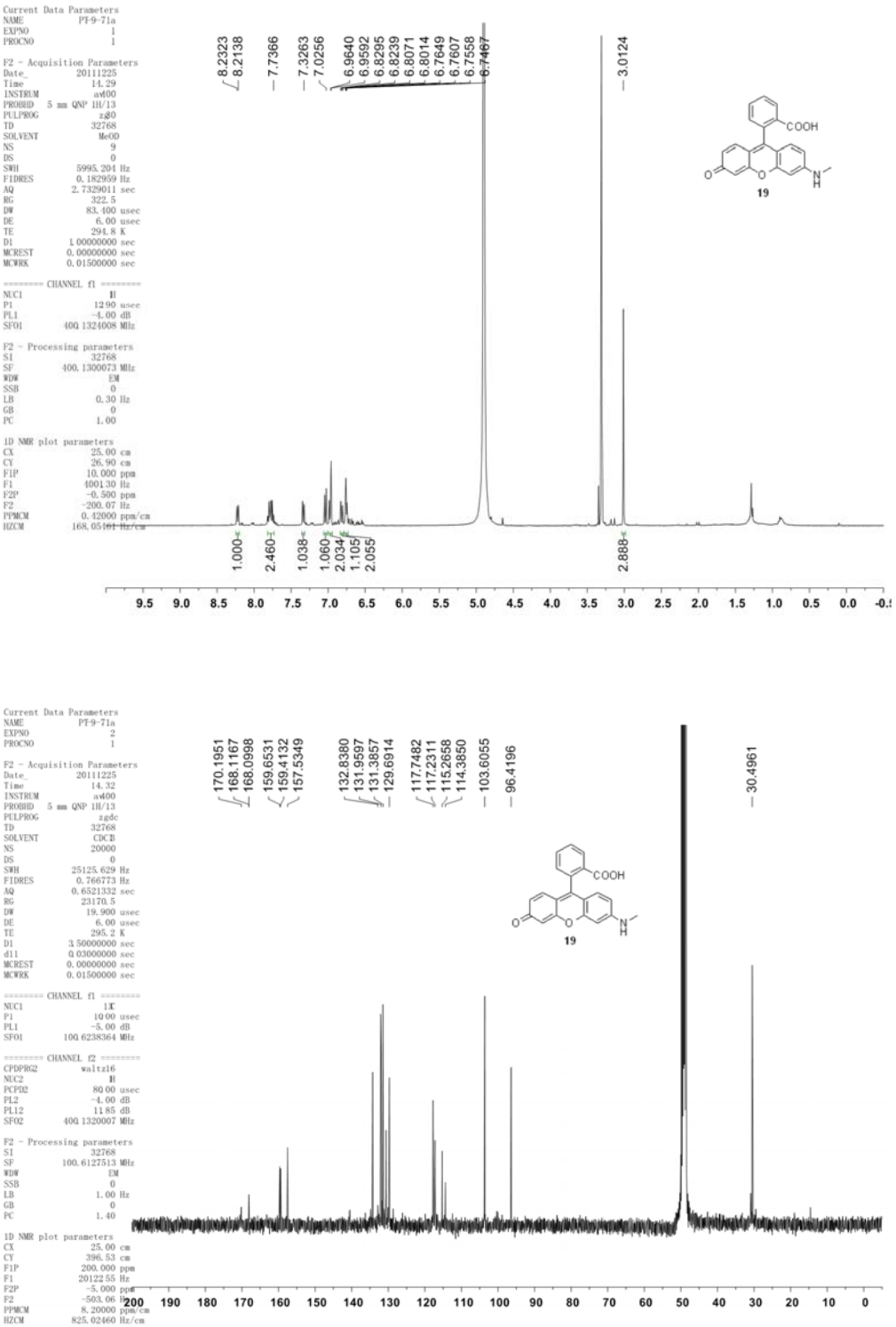

Figure S27. ^{1}H and ^{13}C NMR spectra of compound **19**.

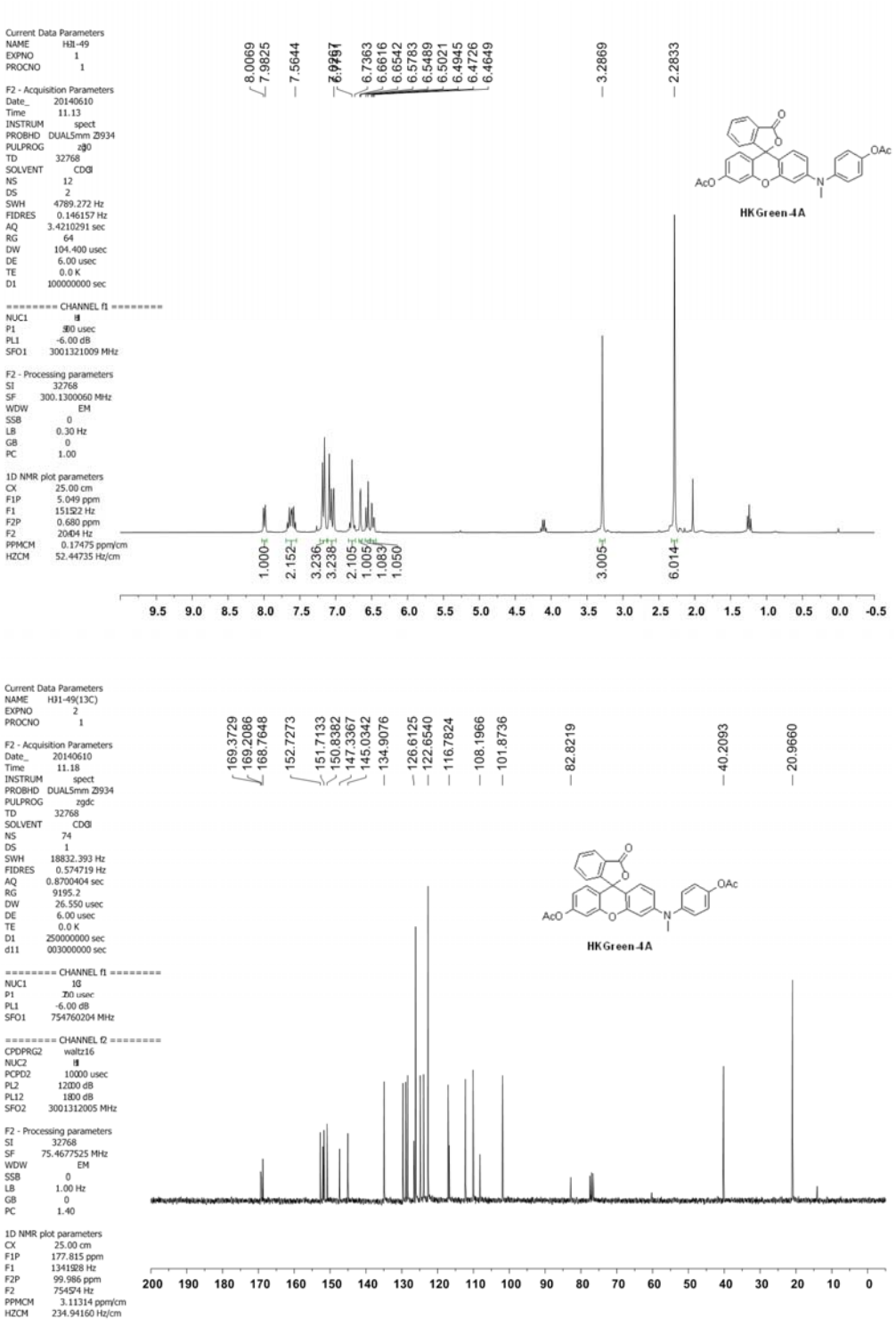

Figure S28. ^{1}H and ^{13}C NMR spectra of **HKGreen-4A**.

9. Reference

(1) Peng, T.; Yang, D. *Org. Lett.* **2010**, *12*, 496.

(2) Pluth, M. D.; McQuade, L. E.; Lippard, S. J. *Org. Lett.* **2010**, *12*, 2318.

(3) (a) Medinas, D. B.; Toledo, J. J. C.; Cerchiaro, G.; do-Amaral, A. T.; de-Rezende, L.; Malvezzi, A.; Augusto, O. *Chem. Res. Toxicol.* **2009**, *22*, 639. (b) Lapenna, D.; Ciofani, G.; Cuccurullo, C.; Neri, M.; Giamberardino, M. A.; Cuccurullo, F. *Free Radic. Res.* **2012**, *46*, 1387.